Studies on antigen presenting cells and T cells in airways and skin

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ABBREVIATIONS

AMP	antimicrobial peptides
Ag	antigen
APC	antigen presenting cell
APC	allophycocyanin
AHR	airway hyperresponsiveness
AMDC	airway mucosal dendritic cells
BALT	bronchus-associated lymphoid tissue
CD	cluster of differentiation
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CHS	contact hypersensitivity
CLA	cutaneous lymphocyte antigen
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte associated antigen 4
Cy2	cyanine 2
Cy3	cyanine 3
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	dendritic cell
DC-LAMP	DC-lysosome-associated membrane glycoprotein
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DDC	Dermal dendritic cell
DNA	deoxyribonucleotide acid
ER	endoplasmic reticulum
FOXP3	forkhead box protein 3
HDM	house dust mite
HEV	high endothelial venule
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IPEX	immunodysregulation polyendocrinopathy enteropathy, X-linked syndrome
LC	Langerhans cells
mAb	monoclonal antibody
MALT	mucosal associated lymphoid tissue
mDC	myeloid DC
MHC	major histocompatibility complex
mRNA	messenger RNA
MxA	Myxovirus resistance protein A
NALT	nasal associated lymphoid tissue
NK	natural killer
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohaemagglutinin
PRR	pattern-recognition receptor
RORγt	retinoic orphan receptor
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
TGF-β	transforming growth factor-β
T_H	T-helper
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
T_{reg}	T regulatory cells
TSLP	thymic stromal lymphopoietin
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

PAPERS INCLUDED

The presented thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

I. BRONCHIAL RESPONSE PATTERN OF ANTIGEN PRESENTING CELLS AND REGULATORY T CELLS IN CHILDREN LESS THAN 2 YEARS OF AGE

Heier I, Malmström K, Pelkonen AS, Malmberg LP, Kajosaari M, Turpeinen M, Lindahl H, Brandtzaeg P, Jahnsen FL*, Mäkelä MJ*

***Thorax.* 2008 Aug;63(8):703-9.**

* These authors share senior authorship

II. CHARACTERIZATION OF BRONCHUS-ASSOCIATED LYMPHOID TISSUE AND ANTIGEN PRESENTING CELLS IN CENTRAL AIRWAYS OF CHILDREN

Heier I, Malmström K, Lohi J, Sajantila A, Mäkelä MJ, Jahnsen FL

Manuscript 2010

III. SUN EXPOSURE INDUCES RAPID IMMUNOLOGICAL CHANGES IN SKIN AND PERIPHERAL BLOOD IN PSORIASIS PATIENTS

Søyland E *, Heier I [*], Rodríguez-Gallego C, Mollnes TE, Johansen F-E, Holven KB, Halvorsen B, Aukrust P, Jahnsen FL, Krogstad A-L, Nenseter MS

* These authors share first authorship

Paper submitted 2009

IV. SUN EXPOSURE RAPIDLY REDUCES PLASMACYTOID DENDRITIC CELLS AND INFLAMMATORY DERMAL DENDRITIC CELLS IN PSORIATIC SKIN

Heier I, Søyland E, Krogstad A-L, Rodríguez-Gallego C, Nenseter MS, Jahnsen FL

Paper submitted 2009

1. INTRODUCTION

1.1. Innate and adaptive immunity at epithelial surfaces

The skin and mucosal surfaces represent large and vulnerable interfaces between the host and a potentially hostile environment and a competent immune system is essential to survival. In vertebrates the immune system consists of two arms - the innate (natural) and the adaptive (acquired) immune system (1). The former is evolutionary ancient, rapid but has limited antigen specificity. It has no memory function and its efficiency does not improve during a response (2). It includes humoral or soluble components like the highly potent complement system, primarily active against extracellular pathogens; type 1 interferons (IFN) produced by virusinfected cells, inhibiting further spread of intracellular pathogens and substances with non-specific antimicrobial activity, such as lysozyme and antimicrobial peptides (AMP), secreted on epithelial surfaces (2). The cellular components of the innate immune system efficiently capture and destroy invading pathogens. They include short-lived granulocytes which kill microbes through phagocytosis and secretion of AMPs (neutrophils) or through release cytotoxic granules contained in their cytoplasm (eosinophils and basophils) (1; 2). Natural killer (NK) cells recognize virusinfected cells and kill them through cytotoxic granzyme and perforin (3). Macrophages and dendritic cells (DCs) represent a particular subset termed antigen presenting cells (APCs). These cells also eliminate pathogens but have a vital additional role in alerting the adaptive immune system.

The adaptive immune system consists of T and B lymphocytes. This phylogenetically younger part of the immune system is capable of mounting highly specific responses, but is temporally delayed. Lymphocytes are primarily activated through clonally distributed receptors that are highly antigen specific. Development of T cell receptors (TCR) and B-cell receptors (BCR), involves specialized DNA rearrangements, through recombination from preexisting V(D)J gene segments (4). This process is controlled by the activitiy of recombination activation genes (RAG), which allow the generation of up to 10^{15} different antigen specific receptors (1). The system improves in both quality and quantity during an immune response through mechanisms involving clonal selection and expansion. Additionally, it maintains a pool of memory T and B cells after the initial immune response has subsided, thus generating immunological memory, important for a faster and more robust recall response (5; 6).

The BCR is surface-bound immunoglobulin that recognizes native antigen, derived from protein, polysaccharide or lipids. B cells may be activated through engagement of the BCR but need T-cell help in order to generate an effective antibody response. Activated B cells differentiate into antibody-producing cells, called plasma cells. Antibodies are soluble immunoglobulins that are secreted from plasma cells. Antibodies that bind to microbial surface antigens mediate killing of microbes by complement activation or by enhanced phagocytotic activity of neutrophils, macrophages and DCs (7).

The TCR can only recognize and bind antigen that is presented as immunogenic peptides in the context of major histocompatibility complex (MHC)-I or MHC-II molecules by APCs (7). All nucleated cells in the organism express MHC class I molecules, which are recognized by $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs). CTLs are particularly important in defence against viral infections. They recognize target cells that express virus-derived peptide on MHC-I molecules and kill them via the cytotoxic molecules granzyme B and perforin, thus limiting spread of the infection (7). Professional APCs also constitutively express MHC class II, specialized for presenting processed peptides to cognate $CD4^+$ T helper (T_H) cells.

The skin and the mucosal tissues are populated both by cells of the innate immune system, as well as effector T and B cells. T and B cells enter the skin or mucosa after having been activated by APCs in regional secondary lymphoid tissue, like lymph nodes. Secondary lymphoid tissues represent so-called inductive sites, i.e. the locations where adaptive immune responses are initiated. The microanatomy of these structures ensures that immune cells from peripheral organs and blood are brought together in close proximity in order to exchange information about a potential threat to the organism. The gut mucosa contains constitutive lymphoid aggregates functioning as inductive sites (the Peyer's patches, multiple isolated lymphoid follicles and the appendix), and together with mesenteric lymph nodes these structures are collectively described as mucosa associated lymphoid tissue (MALT) (8). MALT in the airway mucosa consists of organized lymphoid tissue in the upper airways, i.e. Waldeyer's ring and nasal associated lymphoid tissue (NALT) and draining lymph nodes along the respiratory tract. The central airways also harbour bronchus associated lymphoid tissue (BALT) under certain conditions (see below) but the function of these structures in humans is poorly understood.

1.2. Immunobiology of antigen presenting cells

1.2.1. Dendritic cells

APCs are defined as cells being able to present antigen to T cells. They are characterized by the expression of MHC-II molecules and include B cells, macrophages and DCs. The most important and the best described professional APC population are the DCs, which possess the unique ability to induce immune responses in naïve T cells (9; 10). Steinman and Cohn first described DCs in 1973, when they discovered cells with long branch-like extensions (from Greek *déndron*, "tree"), in lymphoid tissue (11). The development of long dendrites in their mature form creates a large surface at which communication with T cells may take place. DCs are decision-makers, determining whether or not to initiate an adaptive immune response. The molecular interactions between the DC and the T cells recognizing the peptide it presents, determine the magnitude and quality of that immune response (10). Thus, these cells fulfil the vital task of bridging the innate and adaptive immune systems.

In addition to their role as APCs, DCs produce cytokines which allow them to exert effector cell functions. A subset of DCs called plasmacytoid DCs (pDCs) produce large amounts of IFN-α, important in anti-viral defence (12). Also, DCs have recently been found to function as NK cells, inducing apoptosis in target cells in cancer as well as in infection with the intracellular bacterium *Listeria monocytogenes* (13; 14).

1.2.2. Activation and maturation of DCs

DCs are present in blood and all tissues but accumulate in strategically important locations: the interfaces between the host and the environment like skin and mucosae. Here, under steady state conditions, they exist in an immature form, with few dendrites and low levels of MHC-II molecules. They function as sentinels, constitutively sampling foreign antigen as well as endogenous material, (e.g. apoptotic cells) from their environment (10; 15). The concept of DCs residing in peripheral tissues in an immature form, capturing antigen, migrating, maturing and presenting antigen in regional lymphoid tissues was first described for the epidermal Langerhans cells, and was recently referred to as the "Langerhans cell paradigm" (16) as summed up in figure 3.

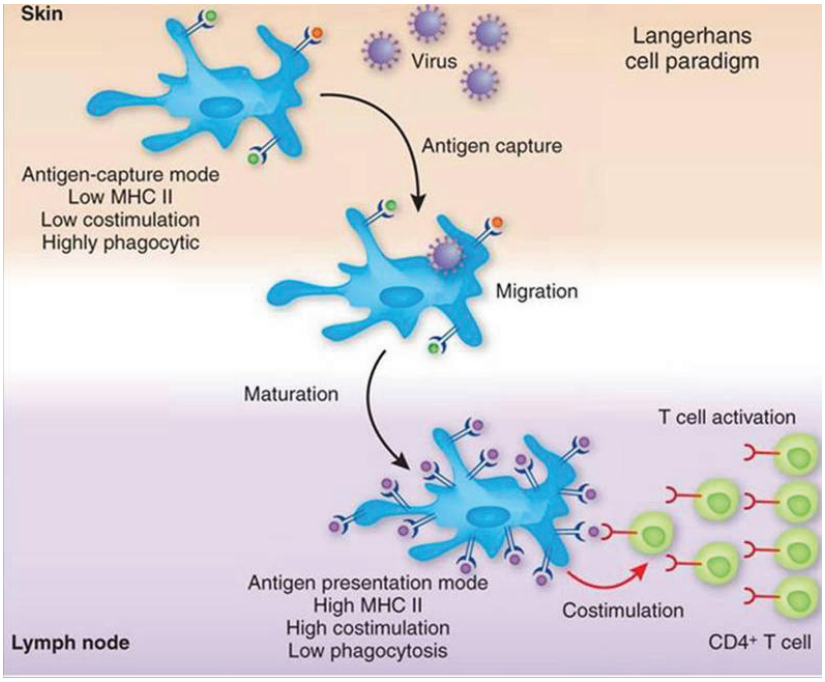

Figure 1. The Langerhans cell paradigm.

DCs spend the skin phase in antigen-capture mode, but after encountering pathogen, they migrate to the draining lymph node and mature by upregulating costimulatory molecules and MHC class II–antigen complexes so they can activate naive T cells.

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APCs are alerted to the presence of potential pathogens through specialized surface receptors, called pattern-recognition receptors (PRRs). These receptors may mediate uptake of extracellular material (see below) as well as activation signals (2). PRRs are germ-line encoded and highly conserved through evolution, i.e. they are found in similar forms in all vertebrates, recognizing molecular patterns common to all microbes (17). Bacteria, viruses, fungi and protozoae carry molecules on their surface which have been unchanged for millions of years, like lipopolysaccarid, peptidoglycan, flagellin, unmethylated CpG motifs, collectively termed pathogen associated molecular patterns (PAMPs) (17). When APCs have been activated by PRR-ligand interaction they become very efficient APCs expressing costimulatory molecules and cytokines which give additional activation signals to the T cell (see below).

Toll-like receptors (TLR) are the best studied PRR (18). TLRs are mammal analogues of drosophila Toll and vital to the function of the innate immune system (18; 19). 10 different TLRs have been described in humans and they differ in their subcellular location, their use of adaptor molecules and activation of intracellular signalling cascades. TLRs 1,2, 4, 5 and 6 are situated on the cell surface and recognize PAMPs of extracellular microbes, such as the bacterial cell-wall component lipopolysaccharide (LPS), bacterial flagellin, lipoprotein and peptidoglycan (Figure 2). In contrast, TLRs 3, 7, 8 and 9 are located in intracellular endosomal-lysosomal compartments, where they recognize RNA and DNA motifs of intracellular pathogens such as viruses and intracellular bacteria and parasites (19).

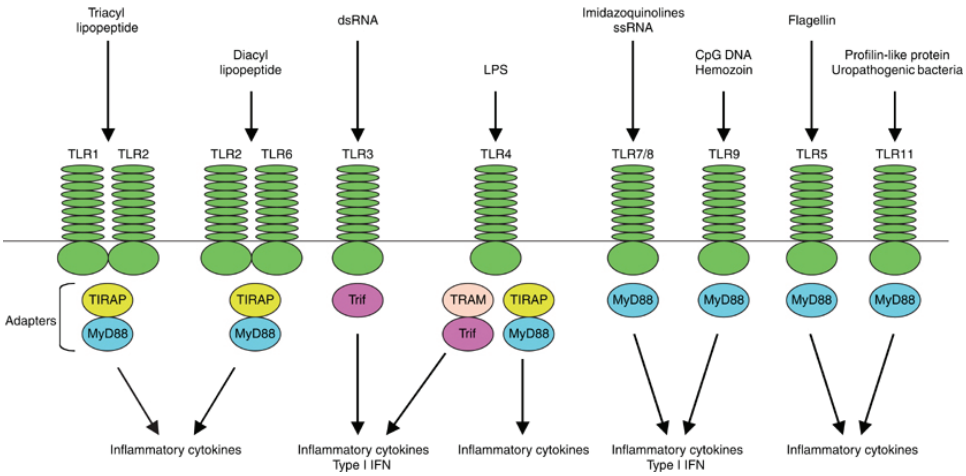

Figure 2. TLR-mediated immune responses.
TLR2 in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl and diacyl lipopeptide, respectively. TLR3 recognizes dsRNA. TLR4 recognizes bacterial LPS. TLR7/8 mediates recognition of imidazoquinolines and ssRNA. TLR9 recognizes CpG DNA of bacteria and viruses. TLR5 recognizes bacterial flagellin and TLR11 (only in mice) recognizes uropathogenic bacteria and the protozoan parasite *Toxoplasma gondii*. TLR1/2 and TLR2/6 utilize MyD88 and TIRAP/MAL as essential adapters. TLR3 utilizes Trif. TLR4 utilizes four adapters, including MyD88, TIRAP/MAL, Trif and TRAM. TLR7/8, TLR9, TLR5 and TLR11 use only MyD88. The MyD88-dependent pathway controls inflammatory responses, while Trif mainly mediates type I IFN responses. In addition, TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner in pDCs

1.2.3. Antigen uptake

Antigens may be endocytosed by a variety of mechanisms (15). Large particulates (bacteria or cells) are often recognized by membrane receptors that trigger the formation of large endocytic vesicles (phagosomes), a process known as phagocytosis. Macropinocytosis is mechanistically similar but in this case the vesicle simply engulfs a large portion of extracellular medium ("cellular drinking"). Both these processes require recruiting the actin cytoskeleton. Another mechanism to engulf extracellular medium is micropinocytosis, which requires the generation of clathrin-coated pits. Finally, receptor-mediated endocytosis consists of the internalization of molecules recognized by specific membrane receptors, which also trigger the formation of clathrin-coated pinosomes (15). Different classes of cell-surface receptors are described, the best known of which are Fc-receptors recognizing immune complexes, C-type lectins (Langerin, DC-SIGN, Dectin-1, mannose receptor and DEC205) recognizing microbial carbohydrates (20), scavenger receptors recognizing apoptotic cells and integrins, recognizing apoptotic cells and opsonized antigen. Uptake of material by macro- and micropinocytosis is often referred to as "fluid-phase" endocytosis to indicate that it is nonspecific rather than being triggered by particular molecular cues intrinsic to the endocytosed material.

1.2.4. Antigen processing, presentation and migration of DCs

Once engulfed by the DCs, the material sampled enters the MCH-II pathway, being first degraded by proteases in lysosomes. The generated peptides are transported into the lysosome-related intracellular compartments which contain MHC-II molecules. The late acidic lysosomes fuses with endosomes laden with MHC II molecules derived from the endoplasmic reticulum (ER) and the antigen-derived peptides are loaded onto the MCH-II molecules. This complex is then transported to the cell surface for presentation to $CD4^+$ T cells as immunogenic peptides located in the groove of the MHC-II molecule (15; 21). In immature DCs, surface MHC-II molecules have a short half-life and are rapidly internalized. Activation of DCs increases the rate of surface MHC II-peptide expression and also decreases the turn-over of these complexes, thus facilitating prolonged availability for cognate $CD4^+$ T cells.

Antigens derived from the intracellular compartment, both endogenous constituents of the cell and pathogen-derived products, are continuously degraded as part of cell homeostasis. Peptides are transported to the ER, where they are loaded on to MHC I

molecules and subsequently transported to the cell surface for presentation to $CD8^+$ CTLs (15). Some DCs also have the ability for so called cross-presentation, i.e. antigen sampled from the extracellular environment can enter the endogenous pathway and be presented on MHC class I molecules, thus generating a CTL-response against antigens not necessarily expressed in DCs (15; 22). Because viruses and some bacteria and parasites are obligatory intracellular pathogens, this pathway is important to alert the adaptive immune system to the presence of such an infection. This pathway is also important in immune responses to cancer (23).

Different pathogens express different PAMPs, and the combination of these PAMPs serves as a fingerprint that triggers a specific set of PRRs on DCs, leading to the integration of signaling pathways to tailor the immune response to that specific pathogen (20; 24). TLR-engagement indicates danger and activates down-stream cell signalling cascades, most of them involving the adaptor molecule MyD88 (Figure 2). This results in expression of pro-inflammatory cytokines enhancing further down-stream effects on the adaptive immune response. TLR-signaling initiates a maturation process in the DC, characterized by upregulation of molecules for antigen presentation, i.e. MHC-I and II, and co-stimulation of T cells, such as CD80/86 (also called B7.1 and B7.2) (Figure 3) and CD40. Simultaneously, antigen uptake by phagocytosis and macropinocytosis as well as receptor mediated pinocytosis is downregulated but all endocytic activity is not shut down and mature DCs retain some antigen sampling activity (15). Endogenously produced molecules released at sites of ongoing inflammation, socalled damage associated molecular patterns (DAMPs), may also activate DCs through TLRs or other surface receptors (25; 26). DCs express several DAMP-receptors, such as protease-activated receptors (PARs) (27), C5a and C3a anaphylatoxin receptors (28), prostaglandin receptors (29) and purinergic receptors sensing extracellular ATP (30). DCs can also be activated by various pro-inflammatory cytokines produced by other cells, such as IL-1 and TNF-α, although in order to become fully capable of initiating an adaptive immune response, direct TLR-signalling is necessary (25; 31).

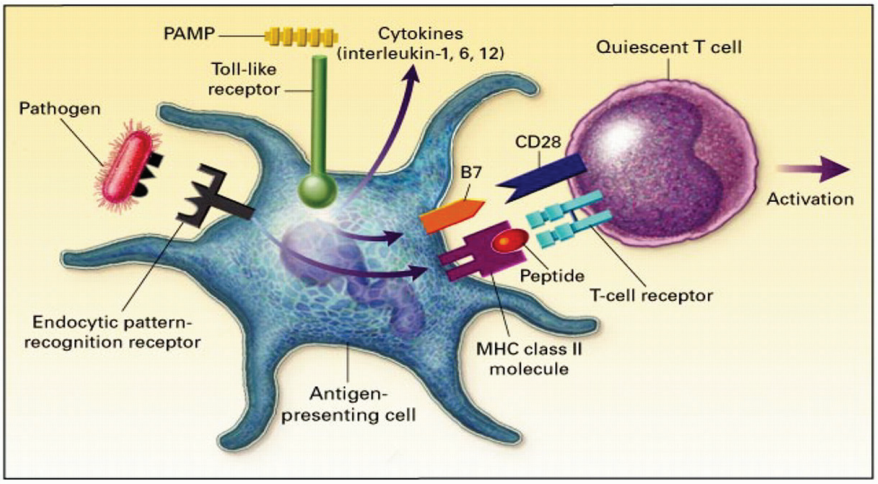

Figure 3. The receptors involved in the interplay of the innate and adaptive immune systems. Recognition of the pathogen-associated molecular pattern (PAMP) by pattern-recognition receptors, such as the toll-like receptors, generates signals that activate the adaptive immune system. Endocytic pattern-recognition receptors bind to components of microbial cell walls and mediate the uptake and phagocytosis of pathogens by antigen-presenting cells (macrophages and DCs). Proteins derived from the microorganisms are processed in the lysosomes to generate antigenic peptides, which form a complex with major-histocompatibility-complex (MHC) class II molecules on the surface of the APC. These peptides are recognized by T-cell receptors. In the case of the signaling class of pattern-recognition receptors, the recognition of pathogen-associated molecular patterns by toll-like receptors leads to the activation of signaling pathways that induce the expression of cytokines, chemokines, and costimulatory molecules. Therefore, pattern-recognition receptors have a role in the generation of both the peptide–MHC-molecule complex and the costimulation required for the activation of T cells.

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Upon activation and maturation, surface expression of chemokine receptors on DCs is altered. Chemokines are small chemical substances that function as leukocyte chemoattractants. Produced locally, they direct the migration of target cells against a concentration gradient (32; 33). Activated DCs upregulate the chemokine receptor CCR7, which recognizes the stroma-derived chemokine CCL21, expressed in lymphoid tissues. This receptor-ligand interaction facilitates the migration of tissue-resident DCs from peripheral tissues to the T-cell areas of draining lymph nodes (34).

1.2.5. Activation of $CD4^+$ T cells

Naïve T cells traffic the T-cell regions of secondary lymphoid organs, such as the spleen and peripheral lymph nodes. After arrival in the lymph node, DCs move about within T cell areas, making transient contact with a large number of naïve T cells and more sustained contact with those that recognize antigens presented on the DC surface (35).

Interactions between surface molecules on DCs and cognate T cells create an interface at which signalling between the two cells takes place, termed the immunological synapse. Three types of signals from the DC are required for full activation and polarization of T cell responses (Figure 4).

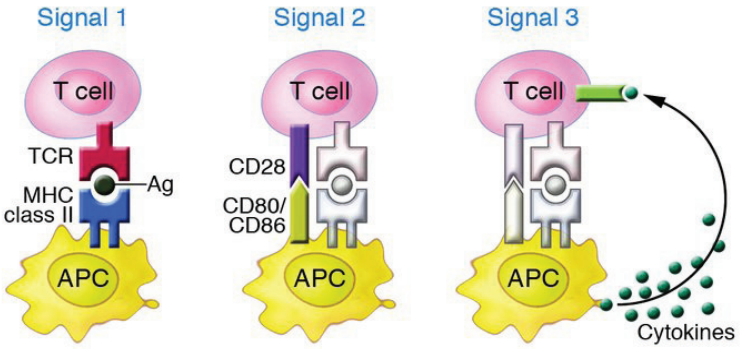

Figure 4. Signal 1, 2 and 3.

Within the immune synapse formed between APCs and T cells, three signals are required for antigen-specific T cell activation. Signal 1 comprises the presentation of antigen peptide, in the context of MHC class II molecules, which is recognized by the antigen-specific TCR. Signal 2 involves the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. CD80/CD86 on APCs interact with their receptor, CD28, on T cells to generate activatory signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals (not shown). Signal 3 is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to polarize them toward an effector phenotype. Ag, antigen.

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The antigen derived peptide presented in the groove of the MHC II-molecule is recognized by the TCR and provides **signal 1** to the naïve $CD4^+$ T cells, conferring information about antigen specificity. Costimulatory molecules on DCs upregulated as part of the maturation process, the best studied of which are CD80 and CD86, interact with CD28 on T cells and provide **signal 2**, required for full activation of naïve T cells. **Signal 3** is given by the cytokines produced by the DC and induces polarization of naïve CD4 T cells into various subsets of effectors (36). The efficiency of T-cell activation depends on the density of DCs

and amount of antigen-MHC-complexes (37) as well as the level of costimulation. All signals appear to be required for full effector T-cell generation (36). Some costimulatory molecules have recently been shown to confer polarization signals, like OX40 for T_H2 polarization (38) and ICOS for T_{reg} induction (39).

DCs translate information about the invading pathogen into a cytokine-gene expression-profile that directs the appropriate T cell differentiation pathway (36) (Figure 5).

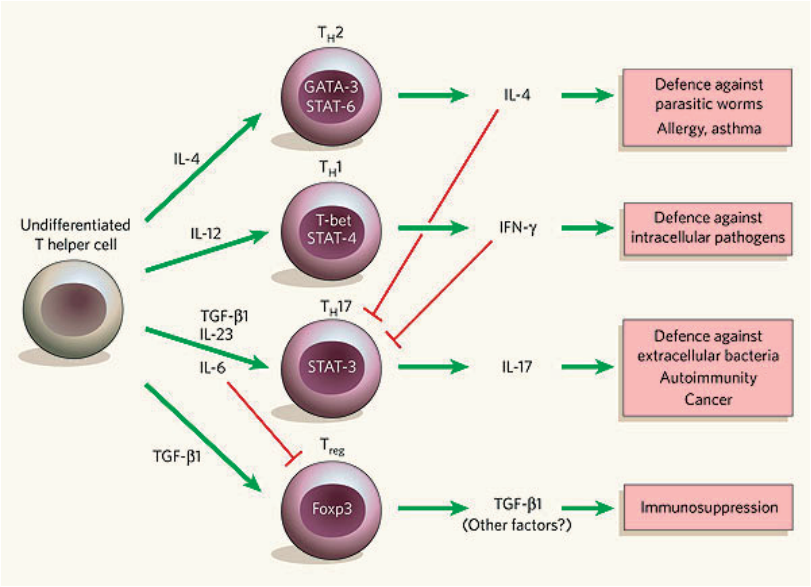

Figure 5. Induction of T cell subsets.

Prompted by different types of interleukin (IL) produced by DCs and other sources, undifferentiated T helper cells can develop into the T_H1 or T_H2 lineages. In an inflammatory response, TGF-β1 and IL-6 promote the development of another lineage, T_H17 cells that produce IL-17. In contrast, interferon-γ (IFN-γ) and IL-4, products of T_H1 and T_H2 cells, inhibit T_H17 differentiation. TGF-β1 boosts expression of the IL-23 receptor, promoting expansion of T_H17 cells by IL-23. But TGF-β1 also promotes the development of another lineage — regulatory T (T_{reg}) cells — by inducing the transcription factor Foxp3, an outcome that is inhibited in the presence of IL-6. Development of T_H1 and T_H2 cells depends on specific STAT proteins and other gene-transcription factors such as T-bet and GATA-3. STAT-3 is probably involved in T_H17 differentiation, and RORγt (not shown) has recently emerged as another T_H17-lineage-specific factor.

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In the presence of intracellular microbes, such as viruses, intracellular bacteria and parasites, DCs produce IL-12 and type 1 IFNs (10). This results in induction of T_H1 cells characterized by the production of IFN-γ. Activated T_H1 cells help to activate macrophages and $CD8^+$ CTLs. Unregulated activation of these responses may result in immunopathology and autoimmune disorders.

Whereas the link between DCs and T_H1 responses is well defined, the mechanisms that induce T_H2 responses have been less clear, as DCs do not produce IL-4, the main T_H2-inducing cytokine (40). T_H2 cells produce IL-4, IL-5, IL-9 and IL-13, stimulating IgE production, as well as eosinophil- and mast cell-differentiation. These factors are important in combating extracellular microbes but are also involved in allergies (41). Recently it was shown that initiation of T_H2 responses was dependent on basophils. These cells were able to present antigens to naive T cells concomitant with IL-4 production, initiating T_H2 responses towards antigens with protease activity (42). Thymic stromal lymphopoietin (TSLP) has also been shown to play an important role in conditioning DCs to induce T_H2-responses (43).

T_H17 cells are characterized by the production of IL-17, IL-22 and IL-6 (44). T_H17 differentiation depends on the presence of IL-6, IL-23 and low levels of TGF-β in mice (44), whereas in humans T_H17 cells are induced by IL-21 and TGF-β (45) and possibly some other combinations of cytokines (46). This cell subset stimulates phagocytes to clear extracellular microbes, but have also been implicated in the pathogenesis of several autoimmune diseases (47; 48). Recently a fourth effector T_H subset has been defined: Follicular Helper T cells (T_{FH}), a subset dedicated to supporting B-cell maturation within lymphoid follicles (49).

All these qualitative different responses are highly efficient when activated under specific inflammatory conditions but are in need of tight control in order to minimize collateral damage. During steady state conditions, i.e. no danger signals present, DCs and macrophages constitutively phagocytose apoptotic cells and present innocuous and self antigens to T cells in an immature form (50). Antigen presentation to naïve T cells with low levels of costimulatory molecules and high levels of the immunosuppressive cytokines TGF-β and IL-10 results in the induction of regulatory T cells (T_{regs}) with suppressive capacity (see below). These cells are critical in order to maintain immunological homeostasis and minimize collateral damage to host tissues under inflammatory situation (51; 52).

At the molecular level, the differentiation of naïve T cells into specific effector subsets is dependent upon the induction of lineage-specific transcription factors: Tbet for T_H1 cells, GATA-3 for T_H2 cells, RORγt for T_H17 cells and FOXP3 for T_{regs} (Figure 5). T_{FH} cells are dependent on Bcl6 induced by IL-6 and IL-21 (53).

Subsequent to activation of T cells, DCs may in turn receive stimulation signals by effector T cells, primarily through interactions between CD40L on activated T cells and CD40 on DCs, thus creating a positive feed-back loop (54).

1.2.6. Imprinting addressins on T cells

In addition to inducing and tailoring adaptive immune responses to best combat the offending microbe, DCs also help activated T cells orientate in the body. Adhesion molecules are induced on the activated T cells, socalled "addressins" or "homing molecules", which specifically interact with corresponding molecules upregulated on the blood vessels of the organ in which the DC originated. This mechanism ensures that when T cells enter the blood stream after activation, they will "home" to the tissues where the pathogen first made entry (55; 56). Naïve T cells express the adhesion molecule CD62L and CCR7, which restricts their migration to secondary lymphoid tissues (55). In contrast, activated T cells downregulate lymphoid-tissue-homing receptors and upregulate tissue-specific adhesion molecules and chemokine receptors that target their migration to non-lymphoid tissues. This imprinting of tissue-homing properties is best described for the gut and skin. Thus, T cells primed by DCs in Peyer's patches and mesenteric lymph nodes express addressins $\alpha_4\beta_7$ and CCR9, which interact with the adhesion molecule MadCAM-1 and CCL25, which enable them to access the gut mucosa upon reentering the blood stream (57). In contrast, T cells that are primed in peripheral lymph nodes upregulate cutaneous leukocyte antigen (CLA), CCR4 (58) and CCR10 (59). These processes have been shown to be at least partly dependent on local vitamin metabolism. In the gut local vitamin A metabolized by DCs induced gut homing properties in T cells (60), whereas in the skin sunlight induced vitamin D metabolized by DCs induced skin homing of T cells (59). Distinct homing phenotypes for leukocyte trafficking to the respiratory tract have yet to be defined, but recent information suggest the involvement of $\alpha_4\beta_1$-integrin and lymphocyte function-associated antigen 1 (LFA1), which correspond with their counterparts VCAM1 and intercellular adhesion molecule 1 (ICAM1), respectively, constitutively expressed on the vessel wall in the bronchial mucosa (61).

Locally produced mediators, such as chemokines, at the site of an infection or allergen encounter, will increase endothelial expression of selectins to initate leukocyte rolling, followed by the expression of integrins to arrest the leukocyte and assist its passage

into the perivascular space. Extravasated leukocytes will then travel up the chemokine gradient to the site of infection or allergen exposure (55).

1.2.7. DC subsets

DCs are a heterogenous population of bone-marrow derived, highly motile and flexible with respect to both structure and function, changing their *modus operandi* within a short time span. Their precise characterization is made difficult by their dynamic phenotype, with changes in surface markers used for identification as well as function, depending on the situation in which they are examined. DCs may be divided into migratory and lymphoid tissue resident DCs (62) or by their presumed origin (myeloid versus plasmacytoid DCs). This latter classification is the most widely used in humans and this also encompasses functional differences (63).

In peripheral blood, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) have been defined based on the expression of the integrin CD11c (64). mDCs represent the largest DC subset both in blood and peripheral tissues. They are commonly defined as HLA-DR^+ $CD11c^+$ DCs and they carry additional socalled myeloid markers, such as CD33, CD13 and CD11b (64). mDCs are potent inducers of T-cell activation. LCs, a subset of mDCs, are located primarily in the epidermis. They contain Birbeck granules and express CD1a and Langerin/CD207 (65). LCs were among the first DCs described and early studies suggested that they are central in T-cell mediated immunity, such as in contact hypersensitivity (CHS) and herpes simplex infections (66-68). However, the concept of LCs as primarily acting as initiators of immune responses has been questioned recently, as T cell activation can be induced in the absence of these cells (69). In mice, LCs were found to be unable to induce CD8 responses against HSV infection (70). Indeed, studies have indicated that CHS is actually dampened by the presence of LC, thus implicating these cells in tolerance induction (71).

pDCs are $CD11c^-$ and have a plasmacytoid appearance (12). They are characterized by the coexpression of CD45RA and CD123 (α-chain of the IL-3 receptor) (72). pDCs are primarily found in lymphoid organs and are rare in peripheral organs except for the liver (73). They are characterized by their ability to produce large amounts of type 1 interferon (IFN) (the best studied of which is IFN-α) during viral infection (12; 63). In addition to inducing a state of resistance to viral infection in tissue cells, type 1 IFNs also activates various immune cells, thus initiating and orchestrating innate and adaptive antiviral

immunity (63; 74). pDCs are clearly less effective than mDCs in inducing T cell responses (75) and are by some considered to be precursor DCs as they acquire typical DC features only after contact with inflammatory stimuli (72). Production of IFN has also been shown to be central to the role of pDCs in various autoimmune processes (74), including lupus erythematosus (76) and psoriasis (77). However, they have also been implicated in tolerance induction both in mice (78; 79) and human models (80; 81)

pDCs were initially believed to have lymphoid origin, based on the lack of myeloid markers (72) and the expression of some lymphocyte features. However, recently several studies have demonstrated that early common precursors can give rise to mDCs, pDCs as well as monocytes/macrophages in mice (82-84) and humans (85). mDCs and pDCs show differential expression of TLRs. Whereas mDC have been shown to express all TLRs except TLR7 and 9, hence primarily sensing extracellular microbes, pDC express the intracellular TLR7 and 9, associated with virus recognition (12; 86).

1.2.8. Trafficking of DCs

DCs and macrophages are derived from precursors in the bone marrow. Distinct subpopulations of circulating monocytes are thought to give rise to resident tissue DCs and macrophages (73). mDCs accumulate at particularly high numbers near the epithelial surfaces, such as skin and mucosae. In contrast, pDCs enter T cells areas of secondary lympoid tissues through high endothelial venules (HEVs), thus acting more like naïve lymphocytes (73).

In the steady state, the turn-over of tissue DCs varies from days in the airways (87) to weeks for the epidermal LCs (65; 88). Inflammatory stimuli increase this turn-over dramatically (89; 90). Cytokines produced locally at the site of inflammation are carried with the blood stream and stimulate the bone marrow to producing and releasing more DC precursors. The most important cytokines stimulating DC development are fms-like tyrosine kinase 3 ligand (Flt3L), granulocyte-macrophage colony stimulating-factor (GM-CSF), CSF-1 and TGF-β (88). Under inflammatory conditions monocytes are recruited at a higher rate, due to increased expression of chemoattractants and adhesion molecules on endothelial cells (91). Recruited monocytes then differentiate into DCs and several cytokines, including GM-CSF, TNF-α, and IL-4, may play a role in this process (88). Animal studies have shown that DC precursors are recruited to airways through mechanisms dependent on the chemokine receptors CCR1, CCR5 and possibly CX3CR1 both under steady-state

conditions and in response to inflammatory stimuli, whereas CCR2 and CCR6 may be important for DC recruitment during secondary immune responses in allergy models (92).

pDCs do not migrate from peripheral tissues to lymph nodes, following the Langerhans cells paradigm, but may acquire antigen locally and enter lymphatic tissue via the blood stream (73). pDCs are continuously replaced by blood-borne precursors but the exact mechanisms that control pDC trafficking remain to be identified (88).

In contrast to most DCs, LCs are radioresistant and repopulate locally in the steady state, either through self-renewal or through a local hematopoietic precursor that takes residence in the skin throughout life under steady state conditions (93). During inflammation, LCs are repopulated by blood precursors, most likely monocytes (94). Also for dermal DCs, evidence of local self-renewal has been found (95). For the skin, the chemokines MCP and CCL20 interacting with CCR2 and CCR6 respectively (96; 97), have been shown to be important for the recruitment of DCs. This chemokine is highly upregulated in inflamed skin such as in psoriasis, resulting in upregulation of endothelial adhesion molecules, with which circulating DC-precursors interact.

In addition to DCs located in peripheral tissues, which show the classic migratory pattern, termed the Langerhans cell paradigm, there are also resident, non-migratory DCs in the spleen and lymph nodes, although studies on these populations are primarily performed in mice and little is known about their human counterparts (62; 73).

1.3. Macrophages

Macrophages are phagocytic cells of the innate immune system with antigen presenting capacity, present in virtually all tissues. Whereas peripheral tissue DCs are characterized by a migratory behaviour, homing efficiently to T cells zones of lymphoid organs for optimal interactions with T lymphocytes, macrophages are more sessile, exerting their physiologic role on site in peripheral tissues (98). They develop from circulating precursors, believed to be monocytes, which migrate into different tissues during steady-state or in response to inflammation, developing into a variety of long-lived tissue-specific macrophages, like Kuppfer cells in the liver, microglia of the brain and osteoclasts of the bone (98; 99). The role of macrophages in phagocytosis of pathogens is best described. They share many of the cell surface molecules, including some TLRs and are basically capable of the same mechanisms for endocotyosis and phagocytosis as DCs. Upon ingestion of microbes, they

degrade the particles through the generation of inducible nitric oxide synthase (iNOS) and highly reactive oxygen species (ROS) generated through ”the respiratory burst” and produce proinflammatory cytokines (100; 101). Processed antigen may be presented to cognate T cells. Macrophages may express costimulatory molecules but are not generally believed to be able to activate naïve T cells. They may activate effector memory T cells on site in peripheral tissues, which have a lower threshold for activation than naïve cells (5). Macrophages primarily activate T_H1-cells, through production of IL-12. In turn, macrophages may receive activation signals from activated T_H1 cells, through IFN-γ production and CD40L-CD40 interactions. This results in increased efficiency in microbial degradation, particularly important in killing intracellular pathogens like mycobacteria (102).

Macrophages are also central in tissue homeostasis, wound healing, recycling iron, clearing senescent erythrocytes and cellular debris during tissue remodeling. They may produce immunosuppressive cytokines and modulate adaptive immune responses, also contributing to resolution of inflammation (103). Recently a new classification of macrophages has been suggested, taking this variety of functional phenotypes into consideration: i) classically activated macrophages, producing proinflammatory cytokines like IL-12 and TNF-α in response to pathogens and IFN-γ, ii) regulatory macrophages, producing the immunosuppressive cytokine IL-10 in response to apoptotic cells and T_{regs} and iii) wound healing macrophages, exerting homeostatic effects in response to IL-4, produced during tissue damage (98). Importantly, macrophages adapt to their cytokine environment and may change their physiology in response to endogenous stimuli from innate or adaptive immune signals (98). Human macrophages are generally identified by the surface marker CD163, which is an endocytic receptor for hemoglobin-haptoglobin complexes and the intracytoplasmic CD68. However, no specific markers exist as yet, which may identify the different functional phenotypes.

DCs and macrophages are clearly related and it has recently been argued that the distinction of the two populations as separate entities may be artificial (104).

1.4. Regulatory T cells (T_{regs})

In 1995, the concept of a specialized subset of T cells exerting dominant suppressive activities was reborn and eventually generally accepted (105; 106). A number of different

regulatory T cell (T_{reg}) populations have since been described (T_R1, T_H3 and $CD4^+CD25^+$ T cells) and shown to exert modulating effects on the immune system by a variety of mechanisms (107; 108). $CD4^+CD25^+$ T_{regs} commonly express the transcription factor forkhead box protein 3 (FOXP3), which is thought to be crucial for both their differentiation and maintenance of suppressive function (109; 110). The important regulatory role of $FOXP3^+$ T_{regs} has clearly been established through identification of the human X-linked inherited disease designated IPEX (immune dysregulation, polyenocrinopathy, enteropathy, X-linked) syndrome - an early onset lymphoproliferative disease that affects several organ systems (111). Many patients with IPEX syndrome suffer from severe atopic diseases and scurfy mice (mouse homolog of IPEX) also show allergic dysregulation (112). Reactive airways disease has also been noted in children with a defect in FOXP3 giving a less severe form of disease than the IPEX syndrome (113).

$FOXP3^+$ T_{regs} are broadly divided into naturally occurring Treg (nTreg) and adaptive/inducible Treg (iTreg) populations (114). The thymus derived $FOXP3^+$ naturally occurring T_{reg} (nT_{reg}) are thought to be important for the control of autoreactive T cells and thus prevention of autoimmune diseases (115; 116) but they may also contribute to mediating tolerance towards foreign antigen (117). More recently it has become clear that $FOXP3^+$ T_{regs} can develop outside the thymus under certain tolerogenic conditions (117-120) and these represent the adaptive/inducible T_{reg} (iT_{reg}). iT_{regs} are thought to develop from naïve cells in the periphery (114) and also from effector T cell populations (107; 121), exemplifying the plasticity of the T cell response. Peripheral induction of T_{regs} may represent an important mechanism to generate tolerance to exogenous antigens, such as commensal bacteria, food and pollen antigens. Especially at mucosal surfaces, where the immune system encounters innocuous antigen, often in the presence of microbes, the induction of specific tolerance is vital to homeostasis (122; 123). Requirements for the peripheral induction of T_{regs} include suboptimal TCR signalling or a combination of strong TCR signalling and high levels of TGF-β (119; 124; 125). A role for both TGF-β and retinoic acid has been found in the generation of peripherally induced T_{regs} in the gut (120; 126). This retinoic acid is produced by a specific subset of $CD103^+$ DCs in the lamina propria, thus acting as tolerogenic DCs (126; 127).

T_{regs} can suppress the function of effector T cells, B cells, DCs, macrophages, mast cells, NK cells and NKT cells through a variety of different mechanisms (107). Naturally occurring $CD4^+CD25^+$ T cells seem to mediate their suppressive properties primarily through direct cell-cell contact (128; 129), although production of cytokines, in particular

IL-10, TGF-β and recently IL-35 (130) has also been shown to contribute. In addition to direct effects on effector cells, T_{regs} also exert immunosuppressive effects via interactions with DCs, inducing them to become tolerogenic. Expression of the membrane bound inhibitory molecule CTLA-4, T_{regs} has been shown to be vital to T_{reg} function (131) and exerts its effects probably primarily through interactions with CD80/CD86 on DCs (107; 132). In mice, T_{regs} have also been shown to control the number of DCs (133).

1.5. APCs and T_{regs} in disease

1.5.1. APCs in the airways

Like other epithelial surfaces, the airway mucosa is lined with a dense network of APCs, consisting of DCs and macrophages (61). Airway mucosa DCs (AMDCs) are strategically located both within and beneath the surface epithelium, which allows their sentinel function (61). The airway mucosa is constantly exposed to large amounts of foreign antigen, both pathogens and innocuous material like pollen and house dust mite (HDM), common aeroallergens that trigger immune pathology in allergic individuals (61). The regional immune system thus faces a considerable challenge in determining against which antigens an immune response should be initiated. Animal studies have demonstrated a high turn-over rate of AMDCs compared with skin (87). In upper airways of humans, a large fraction of APCs coexpress DC and macrophage markers, as opposed to findings in the skin (134; 135), suggesting that they are newly recruited from the circulation. In previous studies only very few APCs have been detected in fetal and infant bronchi (136). pDCs are found at low densities in the lungs of adults (137) but few studies exist and until now, no studies characterizing these cells in the airways of children have been performed. In mice, a subset of $CD103^+$ DCs express tight junction proteins, like zona occludens-1 and claudin (138) enabling them to penetrate the epithelium and form dendritic extensions into the lumen, where antigen may be sampled (see below), but whether intraepithelial AMDCs in humans express CD103 has not been confirmed.

There is evidence that antigens entering via the respiratory route generally induce tolerance or a low-level T_H2 immune response as the default pathway even in the presence of TLR-signalling (61; 139), making the airways a vulnerable site for development of allergy. However, it is assumed that stronger TLR-stimulation by microbial products may skew the ensuing response in a T_H1-direction (140-142). The continuous DC-mediated

transport of inhaled Ag to the bronchial lymph nodes was shown to be critical for the induction of tolerance to innocuous Ags (143).

The interplay between epithelial cells and DCs, in which epithelial cells prime or "educate" tissue-resident DCs is being increasingly appreciated (25; 144; 145). TGF-β is a molecule with immunomodulatory effects and is produced by epithelial cells and T_{regs} (146). Mice lacking the transcription factor Runx3, involved in downstream TGF-β-signalling, spontaneously develop asthmatic features. This involves increased numbers of lung DCs with a mature phenotype, expressing high levels of MHC-II, OX40L and CCR7 (147).

1.5.2. APCs in asthma

Asthma is a common chronic inflammatory disease of the airways, characterized by airway hyperresponsiveness (AHR) and acute exacerbations driven by T_H2 cells (148). Atopic individuals have a genetic predisposition to T_H2-dominated immune responses and increased risk of developing allergic asthma, hayfever, food allergy and atopic eczema (149). As yet unknown environmental factors have resulted in a dramatic increase in the prevalence of asthma, and the associated allergic diseases primarily in the westernized world in the past few decades (150). Specific IgE- production results in sensitization to common allergens, which drives the pathologic process of allergic asthma (148; 151). The T_H2-associated cytokines IL-4 and IL-13 stimulate IgE-production, IL-5 recruits eosinophils and IL-9 enhances mast cell growth, thus shaping the atopic immune response (148). As upstream inducers of T-cell activation, DCs are essential in priming these T_H2-responses and thus vital to the pathogenesis of this common disease (152).

Presentation of inhaled antigen to naïve T_H cells occurs primarily in the bronchial and lung draining lymph nodes (141; 153; 154) and DC-T-cell interactions at these sites determine the outcome of the resulting immune response. DC-mediated activation of memory T cells has been shown to take place locally in the mucosa (155; 156). Mouse studies have shown that sensitization to airway allergen involved activation of DCs prior to the onset of T_H2-mediated inflammation (157) and that depletion of $CD11c^+$ DCs during allergen challenge inhibited the development of pathologic characteristics of asthma (158).

Epithelial cells in asthmatics have been shown to produce TSLP, a key cytokine in triggering myeloid DCs to become T_H2-inducing (43) (Figure 6). TSLP-activated DCs stimulate naïve T cells to differentiate into proinflammatory T_H2 cells that produce IL-4, IL-5, IL-13 and TNF-α but not IL-10, a T-cell phenotype found in asthmatic airways (38; 159).

This polarizing capacity has been shown to involve the induction of the costimulatory molecule OX40L on DCs. Moreover, TSLP-activated DCs produce the T_H2-attracting chemokines CCL17 and CCL22 which increase recruitment of T_H2 cells to the site of inflammation (38). The polarization of T_H2 cells induced by TSLP-activated DCs is further enhanced by IL-25, produced by epithelial cells, basophils and eosinphils in response to

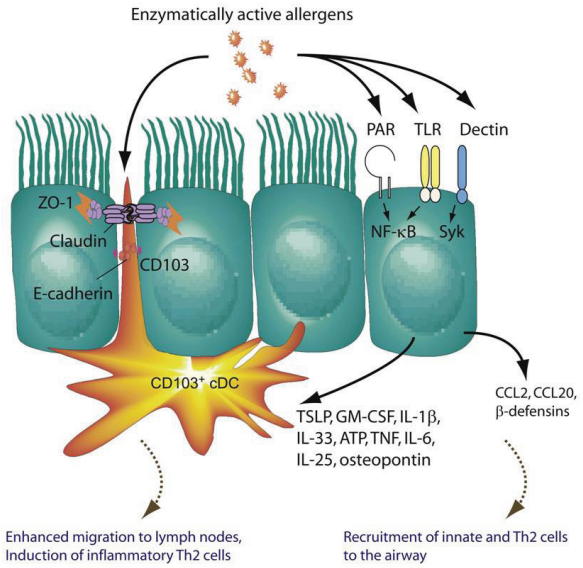

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Figure 6. Interactions between Epithelial Cells and Dendritic Cells in the pathogenesis of asthma

Dendritic cells (DCs) sample the airway lumen by forming dendritic extensions in between epithelial cells. The cells form tight junctions with epithelial cells by expressing occludin and claudin family members as well as zona occludens-1 (ZO-1). In addition, the cells attach to airway epithelial cells via E-cadherin and CD103 expressed by a subset of DCs that probes the airway lumen. Enzymatically active allergens can activate protease-activated receptors (PARs) expressed by epithelial cells followed by nuclear factor-κB (NF-κB) activation and the production of chemokines and cytokines by epithelial cells that attract and activate DCs. Allergens often contain Toll-like receptor (TLR) agonists and C type lectin agonists and triggering through these also induces NF-κB activation and DC activation either directly or indirectly via effects on epithelial cells that also express TLRs and C-type lectin receptors.

allergens (160; 161) and IL-33 (162). Several aeroallergens have been shown to promote T_H2-inducing mDCs, partly through interactions with the epithelium (152; 161). For example, Bet v from birch pollen and Der p 1 and Der p 9 from HDM have intrinsic protease activity (144). This activity has been shown to contribute to impaired T_H1 and enhanced T_H2 immune responses by several mechanisms (144) (Figure 6).

In several mouse models pDCs have been shown to have immunomodulating effects, protecting against asthmatic inflammation (79; 163). Also, in children an inverse relationship between circulating pDCs numbers and the risk of childhood wheezing has been found (164).

1.5.3. T_{regs} in asthma

An increasing body of evidence suggests that T_{regs} are important in modulating the inflammatory process in human asthma (61; 165-167). Children suffering from IPEX, have severe allergic manifestations in addition to widespread autoimmune disorders (111). T_{regs} were found to be impaired in the cord blood of neonates at hereditary risk of allergy (168). Successful allergy immunotherapy is associated with increased number of T_{regs} and elevated levels of IL-10 and TGF-β (169). In several mouse models of asthma, T_{regs} have been shown to be central in ameliorating symptoms. Repeated exposure of mice to low-dose allergen promoted the development of T_{regs}, which could prevent allergic sensitization in naïve mice upon adoptive transfer (170). Induction of T_{regs} has been shown to reverse AHR (171; 172) and adoptive transfer of T_{regs} could suppress allergic inflammation in an IL-10 dependent manner (173). Moreover, T_{regs} were also shown to be able to suppress established inflammation and prevent airway remodelling (174).

1.5.4. APCs in the skin

LCs are the primary APC in the epidermis. As described above, the concept of LCs as primarily initiators of immune responses has been questioned recently. Several studies suggest a role for LCs in the transport and presentation of endogenous skin antigens to the skin-draining lymph nodes under steady-state conditions and thus a role in tolerance induction (175-177).

In the dermis various DC populations have been described, collectively termed dermal dendritic cells (DDCs) (97). In the steady state, the main population of DDCs expresses CD11c and CD1c similar to mDCs in blood. This subset has been termed “resident DCs” as opposed to other DC populations which accumulate under various inflammatory conditions (97). Some DDCs also express the LC marker Langerin and recent studies in mice have shown that these cells are distinct from LCs in the epidermis (65). Only very few pDCs occur in skin under homeostatic conditions (97).

Skin APCs also include distinct populations of macrophages (135; 178). They are characterized by the expression of CD163 and in normal dermis, there is little overlapping expression of this marker and the DC marker CD11c (135).

1.5.5. APCs in psoriasis

Psoriasis is a chronic inflammatory disorder of the skin, which affects 1-3 % of Caucasians. It is associated with severe impairment of quality of life (179) and increased mortality (180). A combination of environmental and genetic factors confers susceptibility to the disease and a dysregulated immune response is central to the pathologic process in the skin (181). Pathological findings are characterized by hyperproliferating keratinocytes, resulting in a severely thickended epidermis (acanthosis) with elongated rete ridges, hyperkeratosis and focal parakeratosis. Cellular infiltrates are found in both epidermal and dermal compartments (181). The disease is widely held to be autoimmune but the autoantigen triggering the inflammatory cascade remains unknown (182). Psoriasis is associated with other autoimmune diseases such as Crohn's disease and rheumatic disorders. Understanding of immune pathology in psoriasis has relevance to other chronic inflammatory conditions, because of shared genetic variants, common immunological pathways and therapeutic targets (183). Unspecific events like local trauma and infections with group A Streptococci may trigger exacerbations (Figure 7).

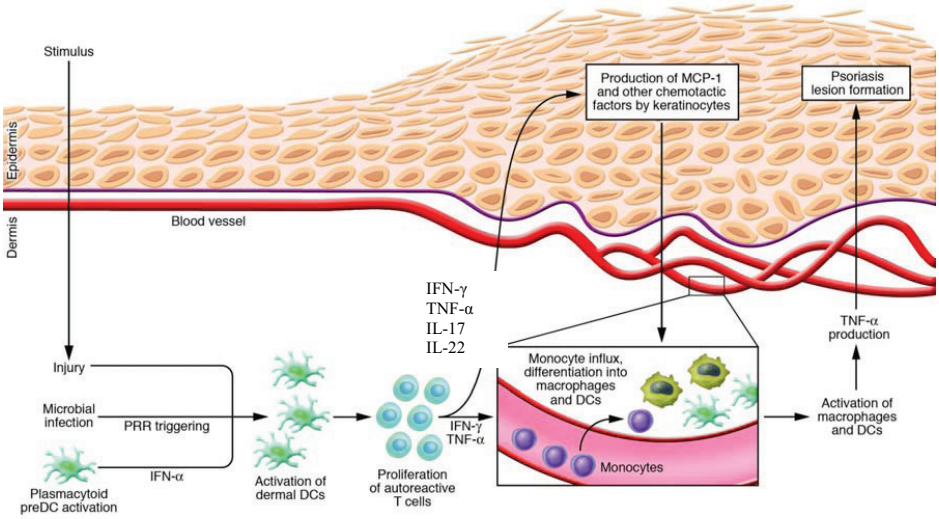

Figure 7. An emerging model of psoriasis pathogenesis in humans.
Many insults can lead to the activation of dermal dendritic cells, a key initiating step in the development of psoriasis in predisposed individuals. Activated dendritic cells induce the proliferation of autoreactive T cells within the dermis, inducing production of IFN-γ, TNF-α, IL-17 and IL-22 which in turn induces the production of MCP-1 and other chemotactic cytokines by epidermal cells. These chemotactic agents induce influx of monocytes from the blood, which undergo differentiation into macrophages and myeloid dendritic cells. Dermal macrophages may, once activated by T-cell or DC-derived cytokines, then produce large amounts of TNF-α, leading to the skin changes observed in psoriasis.

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Immune cell accumulating in psoriatic plaques consist of activated memory populations of skin-homing (CLA^+) $CD4^+$ and $CD8^+$ effector T cells, DCs as well as neutrophils and macrophages (183). Lesional lymphocytes produce T_H1-cytokines, primarily IFN-γ and TNF-α, and the use of anti-TNF-agents is now a well established treatment modality for this disease (184). However, more recently a prominent role for T_H17 cells has been demonstrated (48; 182; 185). Whereas T_H1 and T_H17 cells constitute the proximal cause of immune pathology, through their production of inflammatory cytokines, DCs and possibly macrophages as upstream activators of T cells are the cells believed to initiate this pathologic process. Both mDCs and pDCs are increased in psoriatic lesions (77; 186). In particular, a population of "inflammatory" myeloid DCs expressing CD11c but negative for CD1c is dramatically increased in psoriasis lesions (97; 186). A specialized inflammatory TNF-α and iNOS producing, so-called “Tip-DC” has been described (187; 188). These cells were found to induce proliferation of T cells as well as stimulate the production of T_H1 and T_H17 cytokines (186). It is likely that these Tip-DCs are contained within the $CD11c^+CD1c^-$ mDC population (97). The fact that they produce proinflammatory cytokines supports a role for these DCs as effector cells as well as inducers of T cells (97). Pathogenicity of Tip-DCs in psoriasis is underscored by the rapid downmodulation of their products TNF- α, iNOS, IL-20 and IL-23 during treatment with effective therapies (187; 189). Recently a new therapeutic monoclonal antibody directed against p40, the common subunit of IL-12 and IL-23 has been shown to be effective, underscoring the role for these DC-derived cytokines in driving psoriasis (190).

Although pDCs are a minority of all DCs in psoriasis, they have been shown to be important in driving immune pathology through their production of IFN-α (77; 191). Recently a triggering mechanism was described in which the antimicrobial peptide LL37, which is upregulated in psoriasis, generates a complex with endogenous DNA, activating pDCs through TLR9, thus inducing IFN-α production (192). It was also shown that the same is true for endogenous RNA and these complexes could also activate mDCs via TLR8 and TLR7 (193).

In psoriatic lesional skin, the density of epidermal LCs was found to be lower than in normal skin (194). Also, one study in psoriatic patients found that LCs in non-lesional skin was showed impaired migration upon stimulation with factors that induced migration of LCs in healthy controls (195).

Macrophages also accumulate in psoriatic lesions and have been shown to be a source of pathogenic TNF-α in mouse models of psoriasis (196; 197). There is also evidence that macrophages contribute to psoriasis pathogenesis in humans (198; 199).

1.5.6. T_{regs} in psoriasis

In several autoimmune disorders T_{regs} have been shown to be impaired in their function (200; 201). The inflammatory environment in psoriatic skin favours recruitment and development of pathogenic T cells (182). However, studies have shown that T_{regs} accumulate in lesional dermis along with putative pathogenic effector T cells (202; 203). 80 % of circulating $CD4^+CD25^{high}FOXP3^+$ T_{regs} were found to express the skin homing molecule CLA (204), indicating that T_{regs} are important in maintaining immune homeostasis in the skin. However, a functional study on patients with psoriasis found T_{reg}-populations in peripheral blood as well as in lesional skin to be impaired in their immunoregulatory capacity, suggesting that a malfunction in these cells may contribute to the disorder (205). The proinflammatory cytokine IL-6 renders effector T cells refractive to suppression by T_{regs} (206) and recently it was shown that IL-6 signalling in psoriasis prevented immune suppression by T_{regs} (207). One study also found that $CD4^+CD25^+$ T_{regs} differentiated *in vitro* from $CD34^+$ hematopoietic bone marrow derived cells in psoriatic patients were functionally impaired both with regard to cytokine production and suppressive activities compared with cells from normal volunteers (208).

1.6. Bronchus associated lymphoid tissue (BALT)

BALT is defined as organized secondary lymphoid tissue located within the subepithelial bronchial lamina propria with overlying lymphoepithelium (209). Early studies found that BALT may be present in the human fetal and infant lung (210; 211), but that its appearance is probably dependent on antigenic stimulation (212). Thus, it is not regularly present at birth but may transiently arise during childhood and adolescence (209). A post-mortem study showed that BALT was present in 36.4 % of the patients who had died of SIDS and in 44.1 % of the control cases (213). Whether BALT is a feature of the normal adult lung has not been resolved (214; 215) but is found in various disease states (216). In mice, virus infections induce BALT (217; 218). It was demonstrated that antigen-loaded DCs rapidly

migrate into BALT and efficiently activate antigen-specific T cells and furthermore, $CD11c^+$ DCs were essential for maintaining these structures (218). In a mouse CCR7 knock-out model T_{regs} were shown to inhibit BALT formation (219). In mice lacking spleen, lymph nodes and Peyer's patches it was shown that inducible BALT generated unexpectedly robust primary B- and T-cell responses to influenza virus (220). However, little is known about the function of BALT in humans. The early appearance of BALT in neonatal life suggests that they may be important in establishing mucosal homeostasis in early life (61). Interest in BALT as an inductive site in children is warranted by the potential role of these structures in shaping immune responses both in infections as well as in the advent of effective mucosal vaccinations (221).

1.7. Effects of UV radiation on the immune system

Immunosuppressive effects of UV radiation were first described decades ago. Pioneer studies by Margaret L. Kripke demonstrated that UV-induced tumors transplanted onto normal mice were rejected but when transplanted onto mice treated with immunosuppressive drugs or mice exposed to UV radiation the tumors grew (222). Immunosuppressive effects have been found both locally and systemically (223; 224). In models of CHS, hapten painted on skin areas induces an inflammatory response. However, when the painted area is exposed to low doses of UVB radiation, CHS is inhibited (225). Higher doses of UV radiation also affect immune responses at distant, non-UV exposed sites. CHS cannot be induced in mice exposed to high doses of UV radiation even if the contact allergen is applied at an unirradiated site (226). Mechanisms mediating the immunosuppressive effects have been shown to include both alterations in cellular activitiy as well as the involvement of cytokines. Direct effects on the adaptive immune system include induction of apoptosis in lymphocytes (227). Keratinocytes produce the immunosuppressive cytokine IL-10 in response to UV radiation, which may act both locally and enter the systemic circulation (228). LCs are the best studied DC population in photoimmunology. Epidermal depletion of LC in response to UV radiation is primarily due to migration to regional lymph nodes (229; 230). LCs are impaired in their ability to present antigens through suppression of MCH-II and ICAM-1 expression following UV radiation (231). UV radiation has also been shown to downregulate the costimulatory molecules CD80 and CD86 on both LCs and blood DCs (232; 233). Dermal DCs were shown to

become tolerogenic upon UV exposure (234; 235). Dermal macrophages upregulating IL-10 and downregulating IL-12 were expanded in response to UV exposure (236). Recently the induction of antigen-specific T_{regs} in response to UV radiation has been documented (237; 238). Whereas adaptive immune responses seem to be suppressed by UV exposure, components of the innate immune system are stimulated. UV radiation activates the inflammasome in human keratinocytes, resulting in the processing and release of IL-1β (239). AMPs like cathelecidin are upregulated in the skin upon UV exposure (240).

Exposure to UV radiation triggers the conversion of 7-dehydrocholesterol into cholecalciferol, or vitamin D, thus increasing vitamin D production (241). Vitamin D has multiple modulating effects on the human immune system. Vitamin D inhibits cytokine and chemokine production in macrophages (242), thus preventing excessive inflammation, whereas their "oxidative burst", which increases their ability to kill ingested pathogens is stimulated (243). Vitamin D increases the production of endogenous AMPs in monocytes, neutrophils, and in human cell lines (244-246). Skin DCs metabolizing vitamin D have been shown to induce T_{reg} (59). Topically applied vitamin D3 enhances the suppressive activitiy of $CD4^+CD25^+$ T_{regs} in draining lymph nodes (247). Mechanisms of UV-induced immunosuppression have also been shown to include increased expression of RANKL on keratinocytes, which in turn stimulate DCs to induce T_{regs} (248). One of the primary regulators of RANKL is 1,25 $(OH)_2D_3$, the active form of vitamin D, again showing the importance of vitamin D in modulating immune responses (249; 250).

2. AIMS OF THE STUDY

The aims of the works presented in this thesis were

- Study the organization of the local immune system in the bronchial mucosa of infants and young children related to development of airway obstruction, with emphasis on antigen presenting cells
- Study the early impact of sun exposure on the immunopathology in psoriatic skin, with emphasis on antigen presenting cells and T cell populations.

3. MATERIALS AND METHODS

3.1. Subjects

In paper I the subjects studied were 45 children aged 4-23 months (15 females) under clinical evaluation for respiratory disease. They all had symptoms from the respiratory tract of significant duration (median 60 % of their lifetime, range 19-100 %). Bronchoscopy was performed on clinical indication whereas biopsy sampling was for research purposes. Patients were divided into three groups according to their specific airway conductance (sGaw) based on results from lung function testing with whole body plethysmography: 1) bronchial obstruction with reversibility, 2) bronchial obstruction without reversibility and 3) normal lung function. Reversibility was defined as a 30 % increase in sGaw in response to inhalation of the β2-agonist salbutamol (251). None of the patients had been treated with steroids, either locally or systemically, for the last 6 weeks prior to bronchoscopy.

In paper II, bronchial specimens obtained at autopsy from nine children aged 1.9 – 15.4 years (median 8.5) who died from traumatic causes. No evidence of respiratory disease or atopic eczema were discovered upon autopsy.

In papers III and IV 20 patients (median age 48 years, range 24-65, 6 females) suffering from psoriasis and eligible to receive heliotherapy, a well established treatment modality for psoriasis patients in Norway, were included. They were transported from Norway to Gran Canaria, Spain, in the month of March. The patients were evaluated by the same dermatologist for the Psoriasis Area and Severity Index (PASI) (252) before and after sun treatment. All patients had moderate to severe plaque psoriasis, i.e. mean/median PASI before heliotherapy of 9.8/8.7, range 3.8-18.8. All patients had stopped using any psoriasis medication at least 4 weeks prior to this study. Two of the patients had skin type II and 18 had skin type III according to the Fitzpatrick classification and III. UV doses were measured during the study period (253).

3.2. Airway biopsies

Bronchoscopy of patients in Paper I was clinically indicated, whereas mucosal tissue was obtained for research purposes. Rigid bronchoscopy, which in the hands of experienced operators is as safe as flexible (254), gives a better view on the anatomical structures and

allows obtaining somewhat bigger and better preserved biopsies. Written, informed consent from parents was obtained and the study was approved by the local Ethics Committee. There were no complications associated with the endoscopic procedures. Bronchial biopsy specimens were obtained from the carina, formalin-fixed and paraffin-embedded.

In Paper II, samples were obtained from the proximal main bronchi during autopsy, formalin-fixed and paraffin-embedded.

3.3. Skin biopsies

In Papers III and IV, 4 mm punch biopsy samples were collected from lesional and non-lesional skin in 10 randomly selected patients to evaluate the effect of sun exposure on the local inflammatory infiltrates. The samples from each individual patient were obtained within the same body area at different time points, in sufficient distance to avoid a reactive inflammation from prior biopsy sampling. Specimens were either formalin-fixed and paraffin-embedded or snap frozen in liquid nitrogen and stored at -80˚ C until sectioning.

3.4. Immunohistochemistry

Immunohistochemistry (IHC) is a robust method of identifying antigens (primarily proteins) *in situ* in tissue sections (255). In this method specificity is achieved by applying (mostly) monoclonal antibodies obtained from animals which were immunized by the antigen in question. Tissue sections may be formalin-fixed and paraffin-embedded or frozen in liquid nitrogen, socalled cryosections. Fixation of tissue in formalin induces protein-protein and protein-nucleic acid cross-linking by formation of methylen-bridges (256). This contributes to preserving the morphology of the tissue, but may render antigen inaccessible to Ab binding, socalled “masking”. Formalin-fixed and paraffin-embedded specimens need to be deparaffinized prior to staining. Additionally, boiling of sections is often helpful, socalled “antigen retrieval” (256). In cryosections the microanatomy is often less well preserved, but staining procedures are quicker and antigen masking is less problematic. Frozen samples are placed in a medium called Optimum Cutting Temperature (OCT) for sectioning and fixated in acetone or ethanol before staining.

Several methods for detection of bound antigen in tissue sections exist. Antibodies may be labeled with fluorochromes, which require visualization in a fluorescence

microscope. The antibody recognizing the antigen is called primary antibody and may be monoclonal (recognizing a single epitope or part of the antigen) or polyclonal (recognizing several epitopes). Primary antibodies may be derived from different species (eg. mouse and rat) and be of different Ig subclasses. The different properties of the primary antibodies allows for different mechanisms of detection. After incubating the tissue section with a primary antibody, a secondary antibody is applied, which will interact with the primary antibody, either by using an antibody reacting with immunoglobulin from the specific animal or from the immunoglobulin subclass. Conjugating the secondary antibody with fluorescent molecules, allows visualization of the bound antibody complexes in a fluorescence microscope. Applying primary antibodies of different subclasses (or species) and subclass (or species) specific secondary antibodies with different fluorochromes allows detection of two or three different antigens in the same section (Figure 8).

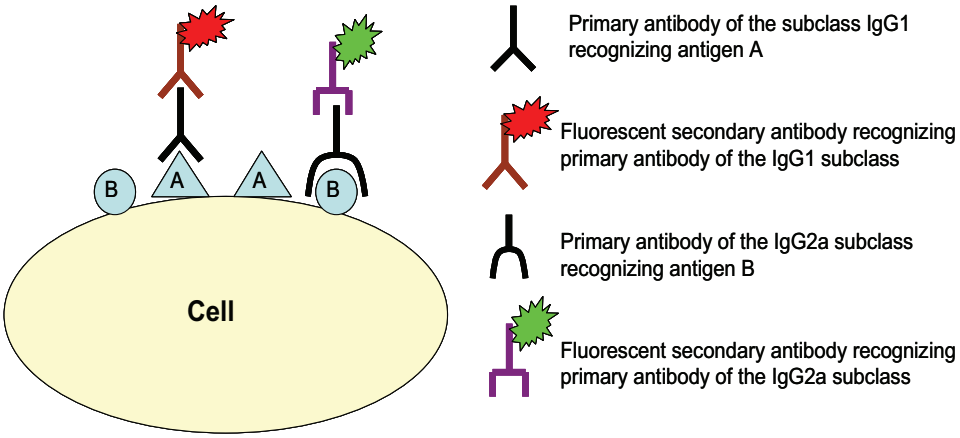

Figure 8. During incubation with the specific primary antibodies, these bind to their respective epitopes. The subsequent application of species- or subclass specific secondary antibodies labeled with different fluorochromes allows detection of several antigens in the same section.

Biotin and the bacterium-derived molecule streptavidin form the strongest non-covalent binding known in the field of biology (257) and this is utilized for enhancing signals in IHC. Streptavidin may be coated with fluorochromes and applied subsequently to incubating sections with biotin-labeled primary or secondary antibodies (Figure 9).

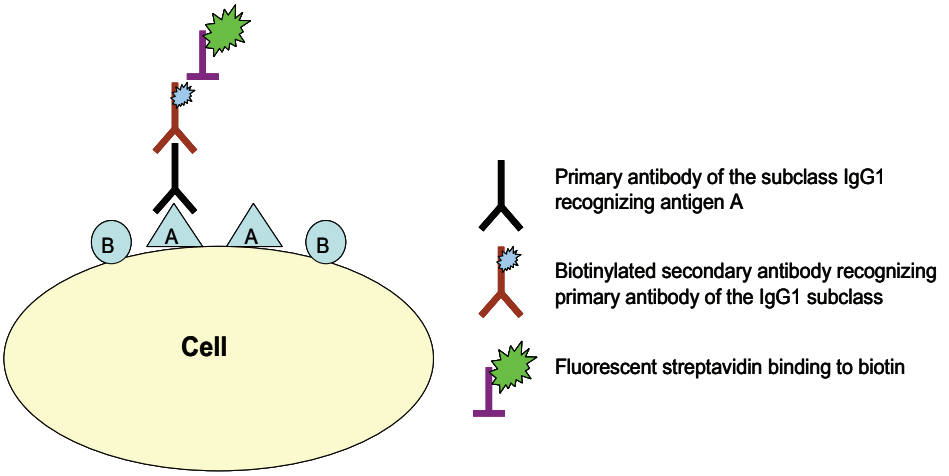

Figure 9. The secondary antibody is biotinylated, allowing for the specific binding of streptavidin labeled with fluorochome in a third step. In tissues with high natural biotin contrent endogenous biotin must be inhibited.

In immunoenzyme staining the secondary antibody is conjugated with an enzyme, eg. peroxidase or alkaline phosphatase (ALP). After incubation steps with the primary and secondary antibodies, substrate is added, which will then change color due to the enzymatic activity and give a staining of the section, allowing visualization by a normal light microscope (Figure 10).

All staining procedures must be performed after careful titration of both primary and secondary antibodies. Positive controls ensure that the staining procedure works and negative controls with irrelevant primary antibodies ensure staining specificity.

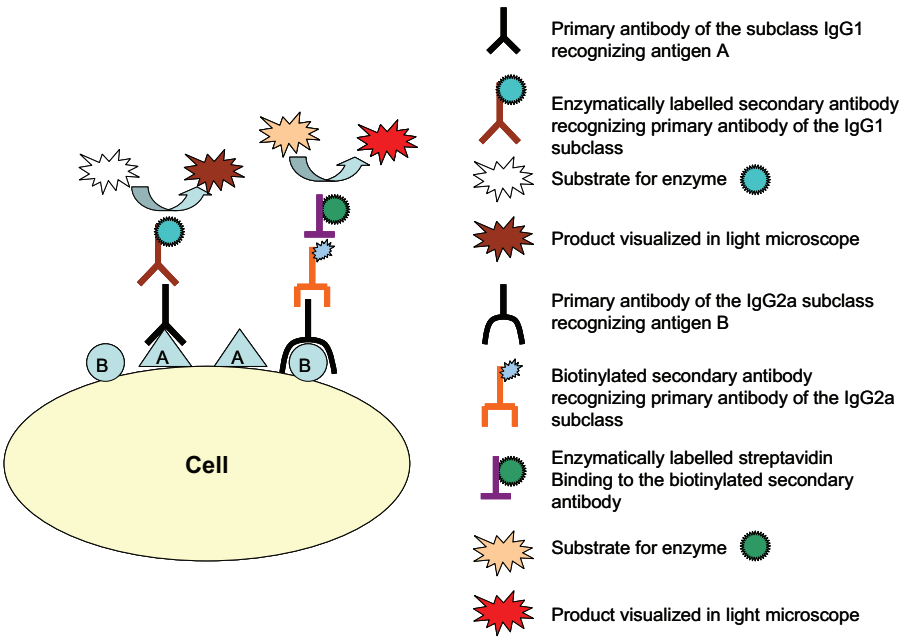

Figure 10. Immunoenzyme staining. The secondary antibody is conjugated with an enzyme (right). After application of the appropriate substrate, a chromogen, will leave a coloured precipitate in the section, which is visible in light microscope. The secondary antibody may also be biotinylated (left) and a different enzyme may be conjugated to streptavidin. Thus requires a fourth step in the staining protocol, but allows for double staining of a section.

Both formalin-fixed paraffin-embedded and cryo-preserved specimens were cut at 4µm and stained with antibodies for IHC. Examination of dewaxed formalin-fixed sections in Papers I and II was performed by immunoenzyme staining in a Ventana NexEs IHC instrument (Tucson, AZ) with the standardized iView DAB or enhanced V-Red (alkaline phosphatase) detection kits as recommended by the manufacturer. For optimal staining results in

formalin-fixed sections that were not stained in the Ventana instrument, heat-induced antigen retrieval was performed by boiling the sections in a microwave oven for 20 min in citrate buffer (pH 6.0) prior to application of antibodies. The primary monoclonal antibodies (mAbs) were directed against human HLA-DR, CD68, CD1a, CD123, CD3, CD20, CD45RA, CD11c and MECA (the latter three only in paper II).

Paired immunofluorescence staining was performed with a polyclonal rabbit anti-CD3 antibody and a rat mAb to Foxp3, followed by Alexa Fluor 488 goat anti-rabbit IgG and Cy3-conjugated donkey anti-rat IgG. Appropriate isotype- and concentration-matched control reagents ensured immunostaining specificity.

Cryopreserved sections were fixed in acetone and kept at -70° until sectioning. In paper III, hematoxylin-eosin (HE) stained sections were evaluated for changes in epidermal thickness. Two-colour immunofluorescence staining was performed combining rabbit polyclonal anti-CD3 with mouse anti-CD4, mouse anti-CD8 or mouse anti-FOXP3. Staining for LCs was performed with anti-CD1a. The primary antibodies were followed by fluorescence-labelled secondary antibodies: Alexa 488 goat anti-rabbit IgG or Cy3 goat anti-mouse IgG.

In paper IV, fluorescence immunostaining of APC populations was performed by costaining sections with mouse anti-human mAbs. Costaining of CD11c and HLA-DR was performed with anti-CD11c (IgG1) and anti-HLA-DR (IgG2a) followed by a biotinylated rat anti-mouse IgG2a antibody and then Cy2-conjugated streptavidin and Cy3-conjugated rat anti-mouse IgG1. Costaining of CD11c and other markers was performed with anti-CD11c (IgG2a) and CD1c, CD163, CD14, DC-SIGN or DC-LAMP (all IgG1), followed by biotinylated rat anti-mouse IgG2a and Cy3-conjugated rat anti-mouse IgG1.

3.5. Microscopy

All stained tissue sections were examined blindly by the same investigator (IH) at x 400 magnification using an ocular grid (250µm x 250µm). In papers I and II, the cell density in the respiratory epithelium was recorded as the total number of positive cell profiles per basement membrane length unit (1 mm), whereas the cell number in lamina propria was recorded as positive cell profiles per square millimeter. The mucosal areas containing lymphoid aggregates were omitted from the cell enumeration.

In papers III and IV, cell numbers were recorded, counting positive cell profiles in epidermis and in the papillary and reticular dermis, to a depth of 250 μm. For the grossly thickened epidermis of psoriatic lesions, cell numbers per square millimetre rather than per millimetre of surface epidermis will reflect the cell density more accurately. Data for both dermal and epidermal cell counts are given as cells numbers per square millimetre, in both lesional and non-lesional skin, in order to make comparisons.

For quantification of epidermal MxA-staining (Paper III), an arbitrary scale from 0 through 5 was established in order to grade the intensity.

3.6. Flow cytometry

Flow cytometry is a method in which single cell suspensions are incubated with monoclonal antibodies specific for cell surface markers expressed in different cell populations. Binding of mAbs may be detected with fluorescent secondary antibodies analogous to IHC. A step containing biotin-streptavidin binding may be included. Alternatively, primary antibodies may be directly conjugated with fluorochromes. Cells are analysed in a flow cytometer in which single cells are exposed to laser beams and fluorescence signals are detected. Signals regarding cell size and granularity are also detected and data are analyzed by the flow cytometer software. Staining is always compared with negative controls samples, i.e. samples in which non-binding primary antibodies are applied, ensuring specificity of signals obtained.

In order to examine effects of heliotherapy on circulating T cell populations, blood was drawn from all patients on days 0, 2 and 16 of sun exposure (paper III). EDTA-whole blood was incubated with monoclonal antibodies against leukocyte (CD45) and T-cell markers (CD3, CD4, CD8 and CLA) directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC). Negative control samples were stained with isotypic-matched irrelevant antibodies. After 20 minutes at room temperature in the dark, erythrocyte lysis was performed. Samples were then washed in PBS and cells were examined by flow cytometry. At least 10.000 lymphocytes were gated by forward and side scatter and CD45 expression. The collected data were analyzed using CellQuest Pro software (Apple computer, Inc; Cupertino, CA).

3.7. Cell culture and cytokine measurements

In Paper III, venous blood samples were collected from all 20 psoriasis patients on days 0, 2 and 16 for investigation of effects on immune cells in the systemic compartment. Peripheral blood mononuclear cells (PBMCs) obtained from heparinized blood by isopaque-Ficoll gradient centrifugation were incubated in flat-bottomed 96-well trays at $2x10^6$ cells/mL; 100 µL/well in medium alone (RPMI-1640 containing 2 mM L-glutamine, supplemented with 100 U/mL penicillin and 5% fetal calf serum), or with phytohaemagglutinin (PHA; final concentration 5 µg/mL). Cell-free supernatants were harvested after culturing for 24 hours and stored at -80^{o}C until analysis.

IL-12p40, IFN-γ, IL-17, TNF-α and IL-10 from culture supernatants were measures using a commercial multiplex cytokine assay (Bio-Plex Human Cytokine 8-Plex Panel, Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturers instructions.

3.8. RT-PCR

Polymerase chain reaction (PCR) is a method in molecular biology used to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (258). Primers (short DNA fragments) containing sequences complementary to the target region and the enzyme DNA polymerase (after which the method is named) are the key components that enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, thus initiating a chain reaction in which the DNA template is exponentially amplified. In order to evaluate the production of a given protein in cell or tissue samples, measurement of mRNA is often used in addition to detecting the protein directly via IHC. To detect mRNA, DNA complementary to the RNA socalled cDNA must be generated first, using the enzyme reverse transcriptase (RT). Thus, the method is called RT-PCR (258).

In Paper III RNA was isolated from cryosections obtained from 5 patients before, after 1 day and after 16 days of sun exposure and subjected to examination by RT-PCR, in order to evaluate the local expression levels of cytokines. The primers applied were specific for mRNA for TNF-α, IL-12p40, IL-23p19, IL-17A, IL-10 and TGF-β. See Paper III for further details.

3.9. Statistical analyses

Non-parametric analyses were applied in all papers. In Paper I, for analysis of between-group differences the Kruskal-Wallis and Mann-Whitney tests were applied. For correlations, the Spearman's rank-order coefficient was used. In Paper III the paired Wilcoxon signed rank test was used to examine differences within the same individuals over time and in Paper IV, three time points within the same individuals were analysed with the Friedman's test. In both papers, analyses of PASI-scores were performed with the parametric Student's t-test. $P<0.05$ were interpreted as significant.

SPSS 12.0.1 for windows (Chicago, IL) and GraphPad Prism 4 (San Diego, CA) were used for the statistical analyses.

4. SUMMARY OF RESULTS

Paper I

Heier I, Malmström K, Pelkonen AS, Malmberg LP, Kajosaari M, Turpeinen M, Lindahl H, Brandtzaeg P, Jahnsen FL, Mäkelä MJ.
Bronchial response pattern of antigen presenting cells and regulatory T cells in children less than 2 years of age. Thorax. 2008 Aug;63(8):703-9.

46 children under investigation for respiratory symptoms, aged 4-23 months, were included in this study. They were divided into three different groups based on clinical presentation, i.e. reversible airway obstruction, irreversible airway obstruction or no bronchial hyperreactivity. Mucosal biopsy specimens from the carina were obtained during bronchoscopy. By immunohistochemistry, we performed detailed characterization of the local immune system. We found a well developed extensive network of APCs both within and beneath the surface epithelium. The density of APCs in the lamina propria correlated positively with increasing age. Some APCs extended their dendrites into the airway lumen, as has been previously shown in the gut mucosa. We found BALT in nearly half of the individuals, suggesting that local inductive sites are a normal feature of infant airways. We found no differences in distribution of APC subsets or BALT between the three different clinical groups.

Paper II

Heier I, Malmstrøm K, Lohi J, Sajantila A, Mäkelä MJ, Jahnsen FL.
Characterization of bronchus-associated lymphoid tissue and antigen presenting cells in central airway mucosa of children.
Manuscript

Here, we studied bronchial specimens obtained post-mortem from nine children aged 2-15 years who died from non-inflammatory causes without signs or history of respiratory disease. Total numbers of APCs and macrophages were comparable to what we previously found in Paper I. In contrast to observations in infants less than 2 years old, there was no

correlation between age and cell numbers, indicating that steady-state numbers have been achieved. In all nine individuals isolated subepithelial lymphoid follicles interpreted as BALT, in which myeloid DCs, B cells, naïve T cells as well as pDCs accumulated. pDCs were also found throughout the lamina propria. In conclusion, we show that the density of APCs previously described in infants and younger children remains throughout childhood and that a steady-state level are reached at approximately 2 years of age. BALT is a feature of normal airway mucosa throughout childhood.

Paper III

Søyland E *, Heier I [*], Rodríguez-Gallego C, Mollnes TE, Johansen F-E, Holven KB, Halvorsen B, Aukrust P, Jahnsen FL, Krogstad A-L and Nenseter MS.
Sun Exposure Induces Rapid Immunological Changes in Skin and Peripheral Blood in Psoriasis Patients.
Paper submitted.
*These authors share first authorship

In this study, we examined immunological parameters in 20 patients with moderate to severe psoriasis, who underwent heliotherapy for 16 days on Gran Canaria. We performed immunohistochemistry on biopsy specimens from 10 patients obtained at baseline and on day 16 of sun exposure and from an additional 5 patients on day 2. Biopsies were obtained from both lesional and non-lesional skin. We detected a rapid decline in lymphocyte numbers from both the epidermal and dermal compartments with both $CD4^+$ and $CD8^+$ T cells being significantly reduced after 16 days. In contrast, the number of dermal $FOXP3^+$ T cells, i.e. putative T_{regs}, remained unchanged, suggesting a relative increase in this cell population. The density of epidermal LCs was markedly reduced in lesional compared with non-lesional skin and was only modestly reduced after sun exposure. In non-lesional skin, as expected, LCs were dramatically reduced in response to sun. In peripheral blood, the number of CLA^+ T cells decreased significantly after only 1 day in the sun and remained reduced at day 16. At day 16, PHA-stimulated PBMCs released significantly reduced levels of pro-inflammatory cytokines. Skin biopsies were examined by RT-PCR for expression of

cytokine mRNA and reduced levels of IL-12 and IL-23 were found at day 16. Together, our data showed that sun treatment had systemic as well as local immunomodulatory effects.

Paper IV

Heier I, Søyland E, Krogstad A-L, Rodríguez-Gallego C, Nenseter MS, Jahnsen FL. Sun Exposure Rapidly Reduces Plasmacytoid Dendritic Cells and Inflammatory Dermal Dendritic Cells in Psoriatic Skin.
Paper submitted

In this study, we examined biopsy material from the same patients as in paper III, with emphasis on dermal APC subpopulations. We found increased numbers of pDCs in lesional skin, which were significantly reduced after 16 days of sun exposure. The surrogate marker for IFN-α, MxA, was also reduced at day 16, suggesting that sun had an inhibitory effect on IFN-α production, most likely produced by pDCs. Further, in lesional dermis, the putative inflammatory $CD11c^+CD1c^-$ myeloid DC was 20-fold increased compared with non-lesional dermis. This subpopulation was selectively reduced in lesional skin upon sun exposure, whereas the percentage of $CD11c^+$ coexpressing $CD1c^+$ increased. $CD163^+$ and DC-$SIGN^+$ macrophages were twofold increased in lesional dermis and decreased significantly upon sun exposure. The percentage of $CD11c^+$ DCs expressing the activation marker DC-LAMP (CD208) was significantly decreased in lesional dermis during sun exposure. A substantial proportion of $CD11c^+$ DCs in lesional dermis coexpressed the macrophage markers CD163 and DC-SIGN, whereas no $CD1c^+$ DCs coexpressed these markers. No overlap in the expression of CD11c and CD163 was seen in non-lesional skin.

In conclusion, sun exposure resulted in rapid reductions in APC subpopulations believed to be involved in mediating psoriasis pathogenesis, probably contributing to the clinical improvement observed.

5. GENERAL DISCUSSION

APCs and T cells activated by them are central players in both T_H2-dominated disorders like asthma and T_H1/T_H17-dominated autoimmune disorders like psoriasis. The aim of this work was divided into two related sub-aims. First, we wanted to examine how the immune system was organized in the bronchial mucosa of infants and young children with special emphasis on APCs and investigate whether cell subsets differed in children with or without atopy-related respiratory disease. Next, we wanted to examine the early impact of sun exposure in psoriasis, with respect to APC and T cell populations locally and in the systemic compartment.

During pregnancy, the fetus develops in a sterile environment and the immune system is immature and skewed towards T_H2-responses (259-261). The first years of postnatal life is important in modeling the maturing adaptive immune system (142). Antigenic exposure results in TLR-stimulation and a gradual increase in T_H1 responses, whereas the tendency to mount T_H2 responses is gradually reduced. Thus, this period represents a window of susceptibility, in which allergic sensitization can take place (142). The T_H2-inducing properties of many aeroallergens are well described, and additional genetic susceptibility factors as well as viral infections may contribute to the development of an allergic phenotype (262). Respiratory syncytial virus (RSV), a common airway pathogen which infects more than 90 % of children before the age of 2, has been shown to induce a T_H2 response (263), which in susceptible individuals may contribute to the development of recurrent wheezing (264). The T_H2-dominated atopic disorders, among which asthma is the most severe, are initiated through the activity of APCs, first of all by DCs through their unique capacity to activate and polarize naïve T cells (152). The occurrence and function of mucosal APCs, and DCs in particular, could therefore play a role in the vulnerable first years of life.

In Paper I, examining airway biopsies from 45 children 4-23 months of age, we found that the number of mucosal APC, defined as HLA-DR^+ cells was significantly correlated with increasing age. Further characterization of the APC population showed that approximately 50 % were $CD68^+$ macrophages whereas the remainders were putative DCs. Others have reported very low numbers of APCs in the airway mucosa in the first year of life (136; 265), making it likely that APCs gradually populate this compartment postnatally in response to antigenic stimulation. The study subjects were divided into 3 groups based on

clinical findings, i.e. bronchial obstruction with or without reversibility or normal lung function, with the hypothesis that these clinical groups might differ with respect to numbers and/or distribution of APC subsets. However, no differences could be found between these groups, nor between atopics or non-atopics. The patients studied here were very young and making a certain diagnosis of asthma in this age group is problematic (264). Follow-up of these children to examine whether differences in airway mucosal cell densities would be reflected in different clinical phenotypes at a later stage would therefore be warranted.

The numbers of $CD1a^+$ intraepithelial DCs were found to be significantly increased in the patients who had suffered a recent respiratory infection (Paper I), suggesting an increased influx of cells due to inflammatory stimuli. Infection with Human rhinovirus (HRV) is a well known inducer of asthma exacerbations (266), but we found no correlation with cell numbers in patients with or without HRV positivity on PCR.

All individuals in Paper I were suffering from respiratory disease and biopsies were obtained on clinical indication. For obvious ethical reasons airway biopsies cannot be obtained from healthy children as controls, therefore, we examined airway tissue from children who had died from non-infectious causes and who had no history or signs of airway disease or atopy (Paper II). Although the subjects were older (2-15 years), total numbers of $HLA\text{-}DR^+$ cells were similar to that found in Paper I, with similar fractions of macrophages and putative DC subsets. No correlation between age and numbers of APCs was found in this population. Although the number of patients included in paper II was relatively low (n=9), it seems plausible to propose that the density of airway mucosal APC increases until approximately 2 years of age, and then reaches a steady-state. At approximately 2 years of age the immune system has been shown to become more mature with respect to several parameters (267; 268).

Paper I showed that 50 % of the small biopsies from bronchial mucosa of children 4 to 23 months of age contained BALT. Importantly, in Paper II, using autopsy material we found that BALT was more common and occurred in much higher densities than previously anticipated. Our findings strongly suggest that BALT is a normal feature of central airway mucosa also in older children. Little is known about the function of BALT in humans, but in mouse models isolated mucosal lymphoid follicles have been shown to be sufficient for the induction of protective immunity against influenza virus infection (220). We defined BALT as cellular aggregates dominated by $CD45RA^+$ cells (a marker of naïve T cells), $CD20^+$ B cells, $CD11c^+$ DCs and some relatively few but predictable pDCs. In some of these structures we also detected HEVs ($MECA\text{-}79^+$). Also, $FOXP3^+$ T_{regs} were found primarily

within these lymphoid structures, although a few were also found scattered in the LP (Paper I). Most functional immunological studies have emphasized the importance of DCs migrating to the draining lymph nodes to prime the adaptive immune system. Here, we show that naïve T and B cells are found together with DCs as organized lymphoid tissue just beneath the epithelial surface at high densities. This may suggest that immune responses to aereoantigens in infants may be initiated to a large extent locally and partly independent of lymph node involvement. These structures should therefore be taken into account when studying the airway immune system in infants and its importance for health and disease.

How DCs capture antigens at human mucosal surfaces has not been determined. In papers I and II we demonstrate that intraepithelial APCs send cellular projections between epithelial cells onto the luminal side. In mouse models it has been shown that these "snorkeling" DCs capture luminal antigens in the airways (138) and in the gut (269). This unique intraepithelial position enable airway DCs to directly sample antigens from the lumen and then transport this material to secondary lymphoid tissue for presentation to T cells. Together, our findings suggest that at least two major routes for uptake of aeroantigens should be taken into consideration; both uptake via BALT and via "snorkeling" APCs.

pDCs have been shown to be vital to the induction of tolerance towards aeroallergens in mouse models (79; 163) and low numbers of circulating pDCs were recently found to be associated with a higher risk of recurrent respiratory tract infections and wheezing in children (164). We describe here for the first time the presence and distribution of pDCs within airway mucosa of children. The finding of pDCs and T_{regs} primarily within the inductive sites of the bronchial mucosa, suggest that these cells may contribute to inducing tolerance to environmental allergens, thus playing a role in immune homoeostasis in childhood. Understanding the regional airway mucosal immune system is vital to the work on developing more effective site-specific vaccines for children (8; 221).

Psoriatic lesional skin is densely infiltrated with inflammatory cells. T cells of the T_H1 and T_H17 phenotypes are widely recognized as important effector cells, but infiltrating DC subsets and macrophages are also central to the immunopathology (Figure 6). In Papers III and IV, we describe rapid effects of sun exposure on immunologic parameters, both locally and systemically. Determining the cell densities at three time points during sun exposure (baseline, days 2 and 16), we observed reduction in both $CD8^+$ and $CD4^+$ T cells in lesional skin already at day 2 (Paper III). This reduction was most pronounced in the

intraepithelial $CD8^+$ subpopulation and presumably primarily due to induction of apoptosis (227; 270). In order to examine the specimens for evidence of apoptosis, we stained for cleaved caspase-3, but could not detect any positive signals, in spite of good results in positive control samples. We speculate that one explanation for this could be due to timing and that the product of apoptosis detected by this method may not be optimally expressed at the time points on which our biopsies were sampled.

This reduction in T-cell numbers was paralleled by a reduction in mRNA levels of primarily IL-12p40, IL-23 and IL-17. The decrease in mRNA levels was non-significant due to low number of samples but suggested that cytokines associated with the IL-23/T_H17-axis were reduced, as has been demonstrated in several previous studies (48). The increased IL-10 mRNA levels in lesional skin in 3 out of 4 individuals at day 16 also supports the concept of a change from an inflammatory into a more homeostatic environment upon sun exposure.

We found that the density of epidermal LCs was significantly reduced in psoriatic skin at baseline (Paper III), in line with previous reports (194). Others have reported normal densities of LCs and discrepancies could possibly be explained by different methods of enumerating cells (97). LCs in non-lesional epidermis were near depleted after sun exposure, as expected (230). In contrast, epidermal LCs in lesional skin were only modestly although significantly reduced, a finding that supports the concept that UV-induced migration may be impaired in psoriasis (195). LCs have been implicated in tolerance induction which suggests that this impaired migratory function of LCs in psoriasis, could contribute indirectly to the pathogenesis. However, as LC migration in non-lesional skin was intact, this does not seem to be a primary defect in psoriasis patients, but may be a secondary phenomenon in psoriatic skin.

Among cutaneous DCs, epidermal LCs is the best studied subset with respect to the immunosuppressive effect of UV therapy (223). However recently, it was shown in a mouse model of contact hypersensitivity that dermal DCs were essential for UV-induced suppression in the absence of LCs (235). We confirmed previous reports that $CD11c^+CD1c^-$ DCs were dramatically (20-fold) increased in psoriatic lesional dermis. This cell population has been found to represent an inflammatory subset, that accumulates in psoriatic lesions, produces TNF-α and iNOS and induces T_H1/T_H17 cells (186; 187). Importantly, we found that these cells were rapidly and selectively decreased upon sun exposure (i.e. the total number of $CD11c^+$ cells was not significantly changed), with marked reductions in cell densities already after 1 day in the sun (Paper IV), strongly suggesting that this subset is

central in immunopathogenesis. Simultaneously, the percentage of $CD11c^+$ DCs coexpressing CD1c increased significantly, thus creating conditions more similar to that found in non-lesional skin. This shows that sun exposure affects several populations of immune cells and suggests that the selective reduction in inflammatory DCs is one of the immunosuppressive mechanisms of UV-exposure.

Whereas pDCs are associated with antiviral immunity and immune homeostasis in the airways, their accumulation in the skin is associated with autoimmune pathology in both psoriasis (77; 182) and lupus erythematosus (76; 271), with unregulated production of IFN-α believed to play a central role. We report, to our knowledge, for the first time that the density of pDCs, as well as expression of MxA, was rapidly reduced in psoriasis upon sun exposure. This reduction preceded clinical improvement, further underscoring the pathogenic role of pDCs (Paper IV). However, the seemingly paradoxical finding that in non-lesional dermis, sun exposure resulted in increased numbers of pDCs, but with no concurrent increase in MxA or signs of inflammation, suggests that these cells may also have an immunomodulatory effect in the skin.

$FOXP3^+$ T_{regs} are vital in maintaining immune homeostasis and have been shown to be impaired in number and function in psoriasis (205). $FOXP3^+$ cells were increased in lesional skin along with the other major cell populations (Paper III), which may support the concept of a reduced function but not recruitment in this disease (205). Epidermal $FOXP3^+$ cells were near depleted along with the two main cell subsets. In contrast, dermal $FOXP3^+$ T cells remained unchanged after 16 days of sun exposure, suggesting that the induction of Tregs is one of the mechanisms mediating sun induced improvement in psoriasis. A possible explanation for the differential effect on $FOXP3^+$ T-cell numbers in the two compartments could be that the dermal cells are less susceptible to apoptosis and that newly recruited $T_{regs,}$ reflecting a change in the local cytokine milieu, will be found in the dermis under such circumstances. However, FOXP3 has also recently been shown to be transiently induced in activated effector T cells (125), and functional analysis of these cells in psoriatic skin would have to be performed in order to show that $FOXP3^+$ T cells in the dermis are truly functional T_{regs}.

Sun exposure has been shown to have significant impact on various components of the immune system (223). At the systemic level, we also demonstrate that skin homing CLA^+CD4^+ and CLA^+CD8^+ T cells in peripheral blood decreases significantly in psoriatic patients after only one day of sun exposure (Paper III). Previously, it has been shown that UVB exposure reduces total CLA^+ T cells in the blood of psoriatic patients after one week

(272). The rapid decrease of CLA^+ T cells in peripheral blood could be caused by an increased migration to the skin as a result of non-specific low-level inflammation after acute sun exposure. However, cell numbers in non-lesional skin were unchanged whereas they were markedly reduced in lesional skin after sun exposure. As CLA^+ T cells preferentially circulate to the skin, they become susceptible to apoptosis and thus peripheral numbers would decrease. We hold this to be the most likely explanation for the observed rapid reduction in circulating skin homing T cells. In Paper III, we also provide evidence of functional immunosuppression in the systemic compartment, through the attenuated capacity of *in vitro*-cultured PBMCs to produce cytokines after sun exposure. However, in the systemic compartment, we demonstrate decreased production of both proinflammatory and anti-inflammatory cytokines. One explanation for this could be that downregulation of proinflammatory cascades in the systemic compartment also results in less stimulation of compensatory immunosuppressive pathways. This would argue against a role of IL-10 in the systemic immunosuppression seen after sun exposure (224; 273). The patients in this study had increased vitamin D levels, which may have contributed to immunomodulation (274; 275).

Biological agents have received much attention in the treatment of severe psoriasis. While therapeutic monoclonal antibodies are effective, their use is expensive and long-term data on safety are still lacking (276). Recently, it was shown that etanercept (TNF receptor-immunoglobulin fusion protein) had a significant effect on psoriasis by inhibiting the activity of dermal DCs (189). Importantly, we show here, that sun treatment has at least comparable effect with two weeks of etanercept treatment based on the reduction of PASI-score, epidermal thickness, $CD11c^+$ DCs, $CD163^+$ macrophages, $CD3^+$ T cells, MxA expression, DC-LAMP expression and IL-23 mRNA.

Warm climate and bathing in combination with sun exposure might reduce stress and thereby indirectly improve the psoriasis lesions in addition to the UV-induced effects. However, there is a large body of evidence to suggest that UV exposure under experimental conditions have a very strong immunosuppressive effect, which is compatible with the notion that the reduction in pathogenic DCs and T cells observed in lesional skin in our study was mainly due to sun exposure.

An additional finding in our study was that in inflamed skin a large proportion of the $CD11c^+CD1c^-$ but not $CD11c^+CD1c^+$ DCs coexpressed monocyte/macrophage markers. This further underscores the relative immaturity of these inflammatory DCs (186). In normal non-inflamed dermis $CD11c^+$ mDCs and $CD163^+$ macrophages are distinct

populations (135), whereas in psoriatic skin, double positive cells accumulate (Paper IV). In the upper airways (134) as well as in the gut (Jahnsen FL, unpublished data) there is considerable overlap between macrophage and DC markers also under non-inflammatory conditions. To a lesser extent we found coexpression of DC and macrophage markers in non-inflamed lower airways (Paper II). The turnover of mucosal APC populations is higher in the steady-state than in the skin (87; 93), presumably due to the higher antigen stimulation. Whereas the skin is protected from the environment by the multilayered cornified epidermis, the mucosae are only separated from the surroundings by a single cell layer (gut and lower airways) or a multilayered but non-cornified (upper airways). Cells expressing markers associated with both DCs and macrophages are found in the circulation (277), and may represent precursors en route to peripheral tissue. The risk of both infections and allergic sensitization is high at mucosal surfaces. In cases with epidermal breach of barrier, as seen in atopic dermatitis, the same is true for the skin (278).

Maintaining immunological homeostasis in mucosal tissues and the skin, i.e. avoiding allergic and excessive inflammatory responses, is a task which to a large extent rests with the APC populations as upstream activators of T cells. The studies presented here contribute to understanding the regional immune mechanisms both in the airways and in the skin.

6. CONCLUSIONS

- Infants have a well developed network of APCs in their bronchial mucosa with pDCs primarily present within the BALT
- APCs in the respiratory tract mucosa increases significantly with age but seems to reach steady state levels at approximately 2 years of age
- No differences could be found in the numbers or distribution in these populations with respect to clinical phenotype
- BALT, including T_{regs} and pDCs are present at a very early age
- Clinical improvement as a result of sun exposure in psoriasis is preceded by early rapid changes in $CD4^+$ and $CD8^+$ T cells, with a relative increase in dermal T_{regs}
- Sun exposure rapidly and selectively reduces pDCs and putative inflammatory mDCs whereas the percentage of DCs associated with homeostasis increases

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I

Paper I

BRONCHIAL RESPONSE PATTERN OF ANTIGEN PRESENTING CELLS AND REGULATORY T CELLS IN CHILDREN LESS THAN 2 YEARS OF AGE

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Paper II

II

CHARACTERIZATION OF BRONCHUS-ASSOCIATED LYMPHOID TISSUE AND ANTIGEN PRESENTING CELLS IN CENTRAL AIRWAYS OF CHILDREN

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Characterization of bronchus-associated lymphoid tissue and antigen presenting cells in central airway mucosa of children

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ABSTRACT

Childhood represents an immunological window of vulnerability in which individuals are at increased risk for both serious infections and development of allergic diseases, particularly in the airways. This relative failure in immune regulation in the airways may be due to functional differences at the level of the local mucosal immune system. However, little is known about how the airway mucosal immune system is organized and functions during early age. Here, we characterized the organization of immune cells in bronchial mucosal specimens obtained post-mortem from nine children aged 2-15 years without signs of respiratory disease who died from non-inflammatory causes. In all nine cases isolated subepithelial lymphoid follicles (ILFs) interpreted as bronchus-associated lymphoid tissue (BALT) were found constituting an average frequency of 60 ILFs per cm^2 mucosa. Outside these ILFs dense networks of $CD11c^+$ myeloid DCs, $CD68^+$ macrophages, and $CD3^+CD45RA^-$ memory T cells were found both within and beneath the surface epithelium. Plasmacytoid DCs also occurred in low numbers. Importantly, intraepithelial antigen presenting cells were found to extend cellular projections into the airway lumen. In conclusion, we show that the density and location of antigen presenting cells and T cells are similar to that observed in adults. However, in contrast to adults, BALT appears to be a normal feature of the airway mucosa throughout childhood, suggesting that these structures contribute to regional immunity and homeostasis. Our findings thus indicate that the local immune system in the airways of children have unique features which should be taken into account when studying its role in relation to respiratory immunopathology.

INTRODUCTION

The respiratory tract has a large mucosal surface that is continuously exposed to a range of antigens, both pathogenic microbes and innocuous material, like dust and pollen. The local immune system therefore faces a considerable challenge in order to discriminate between pathogenic antigens that merit rapid elimination and harmless ubiquitous antigens which need to be "tolerated" to avoid chronic inflammatory damage (1). Epidemiological studies have shown that in preschool years individuals are more susceptible to both respiratory viral infections and aeroallergen sensitization than later in life (2; 3). These findings suggest that there is a relative dysfunction of the airway immune system during the first years of life. At birth the adaptive immune response appears to be attenuated and skewed towards production of Th2 cytokines with a relative inability to produce T_H1 immunity (3). This postnatal T_H1/T_H2-imbalance may predispose for development of T_H2-dependent allergic immunopathology and at the same time increase the risk for viral infections due to inappropriate T_H1-responses. However, although there is a T_H2-skewing in early life, the majority of infants do not become sensitized to inhaled antigens, showing that effective regulatory mechanisms exist (4). This suggests that there may be functional differences at the level of the local mucosal immune system in the respiratory tract. However, little is known about how the airway mucosal immune system is organized and functions during early age (1).

Mouse models have shown that airway mucosal dendritic cells (AMDCs) are the main inducers of adaptive immunity in the respiratory tract (1; 5). They are strategically situated at mucosal surfaces where they continuously sample antigen from the environment. Antigen-bearing AMDCs continuously migrate to the draining lymphoid nodes were they present antigenic material to naïve T cells and initiate productive immune responses (5; 6). AMDCs consist of several subsets which are functionally distinct. Classical $CD11c^+$ myeloid DCs have been shown to initiate T-cell responses to both viruses (7) and aeroallergens (8). The rare plasmacytoid (p)DCs, characterized by high expression of CD123, are also important in anti-viral immunity through their ability to produce large amounts of IFN-α (9) but with respect to allergens, this cell population has been associated with induction of tolerance (10-12). The distribution of DC subsets is therefore relevant to local immune functions. This notion in underscored by the fact that in children with a family history of atopy, lower numbers of circulating pDCs was associated with more

frequent and more severe respiratory tract infections, wheezing and a diagnosis of asthma (13).

An apparent difference between the airway immune system in infants compared with adults is the presence of bronchus-associated lymphoid tissue (BALT) (14; 15). BALT is part of the mucosa-associated lymphoid tissue and is defined as organized lymphoid aggregates within the bronchial mucosa in contact with the surface epithelium (15). In mice, it has been shown that adaptive immune responses against influenza virus could be initiated in inducible BALT (16), but little is known about the function of these lymphoid structures in humans. BALT is not regularly found in fetuses, as opposed to the constitutive Peyer's patches of the intestine, but is probably induced by antigen stimulation after birth (17). BALT in adults have primarily been associated with various diseases (15), but we and others have shown that they often are present in the bronchial mucosa during childhood (15; 17; 18). To understand how adaptive response mechanisms operate in the airways of children both the function of AMDC subsets as well as the participation of BALT should be taken into account.

We have recently characterized the distribution of immune cells in a biopsy material from bronchial mucosa of infants less than 2 years of age suffering from respiratory symptoms (18). We found that there was a tight network of antigen presenting cells (APCs) at the mucosal surface and that BALT was present in more than half of the samples. However, for obvious ethical reasons we were not able to obtain biopsies from normal individuals. To characterize the organization of the bronchial immune system at baseline we have performed a detailed characterization of the distribution and functional phenotype of APCs and the occurrence of BALT in the bronchial mucosa of children who died from non-infectious causes and without any history or recent or chronic signs of airway disease.

MATERIALS AND METHODS

Subjects

Specimens from nine children aged 1.9 – 15.4 years (median 8.5) who died from traumatic causes and with no evidence of respiratory disease upon autopsy were examined. Oedema and hemorrhage were seen at the autopsies by the coronary in most of the samples due to the mechanism of death. None of the individuals had signs of atopic eczema. Samples were taken from the lower trachea or the main bronchi close to the carina. In two specimens, the

epithelium was absent. In one specimen the epithelium could only be evaluated in some sections, whereas in the remaining six, the quality of the epithelium was satisfactory.

Immunohistochemistry

Specimens were formalin-fixed and paraffin-embedded. Sections cut at 4µm were examined by immunohistochemistry. For optimal staining results heat-induced epitope retrieval was performed by boiling the sections in a microwave oven for 20 min in citrate buffer (pH 6.0).

Immunoenzyme staining on dewaxed tissue sections was performed in a Ventana NexEs IHC instrument (Tucson, AZ) with the standardized iView DAB or enhanced V-Red (alkaline phosphatase) detection kits as recommended by the manufacturer. The primary monoclonal antibodies (mAbs) were directed against human HLA-DR (clone TAL.1B5, IgG1, DakoCytomation, Glostrup, Denmark), CD68 (clone PG-M1, IgG3, DakoCytomation), CD11c (clone 5D11, IgG2a, Novocastra Lab. Ltd., Newcastle Upon Tyne, UK), CD1a (clone MTB1, IgG1, Novocastra), CD123 (a mixture of clone 7G3, IgG2a, and clone 9F5, IgG1, BD Pharmingen, San Diego, CA), CD45RA (clone L48, BD Immunocytometry Syst., San Jose, CA) and CD20 (clone L26, IgG2a, DakoCytomation). A polyclonal rabbit anti-human CD3 (dilution 1/50; DakoCytomation) was additionally applied.

Stained tissue sections were examined blindly by the same investigator (IH) at x 400 magnification. The epithelium was missing in 2 individuals, and of very poor quality in one, thus epithelial counts could not be performed in all sections. The cell density in the epithelium was recorded as the total number of positive cell profiles per basement membrane length unit (1 mm), whereas the cell number in lamina propria was recorded as positive cell profiles per square millimeter. The total number of positive mucosal cells was recorded per basement membrane length unit by adding the intraepithelial counts and the lamina propria counts to a depth of the grid. On average, 50 visual fields were counted in each specimen. The mucosal areas containing lymphoid aggregates (see Results section) were omitted from the cell enumeration.

Staining for the nuclear antigens FOXP3 and Ki67 with antibodies that normally work well on paraffin-sections were unsuccessful, probably due to post-mortem degradation of nuclear proteins.

RESULTS

Histologial evaluation

H+E staining showed horizontally cut sections through the whole bronchial wall including parabronchial lymph nodes. The morphology in most specimens was very good with a well preserved surface epithelium in 7 out of 9 samples. In some sections the epithelium was partly detached from the underlying lamina propria. There was no accumulation of neutrophils or eosinophils or other signs of ongoing inflammation. In nearly all sections small cell aggregates in contact with the surface epithelium were observed indicative of isolated lymphoid follicles (ILFs; see below).

BALT was found in all subjects

To characterize the putative ILFs further we performed immunostaining experiments applying a range of mAbs to identify subsets of immune cells (Figure 1). All ILFs contained high numbers of HLA-DR$^+$ APCs. (Figure 1A and B). The aggregates were also characterized by accumulation of CD3$^+$ T cells and CD20$^+$ B cells (Figure 1C and D). Most T cells were of the naïve phenotype judged by the dense population of CD45RA$^+$ cells (Figure 1E and F). CD11c$^+$ but not CD68$^+$ cells accumulated in ILF (Figure 1G and not shown). Moreover, small numbers CD123$^+$ pDCs were observed (Figure 1H) and some vessels expressed MECA-79 demonstrating that they were high endothelial venules (HEVs) (Figure 1I). Together, these structures could clearly be defined as BALT.

The numbers of ILFs were counted in sections stained for CD3/CD20 and CD45RA which clearly delineated these structures (Figures 1C-F). Sections from all individuals contained ILFs with a median of 4 per section (range 1 to 11). The density of ILFs was determined by dividing the number with the bronchial circumference (Figure 2). With an estimated median ILF diameter of 200 μm, we calculated that the density of ILFs was approximately 60 per square cm of airway mucosa. This shows that BALT is a prominent feature of central airway mucosa in children from 1-15 years of age.

Immune cell populations outside organized lymphoid tissue

Apart from ILFs, HLA-DR$^+$ cells were evenly distributed both within and beneath the surface epithelium constituting a dense network of large cells with variable morphology. Within the epithelium a median number of 34 HLA-DR$^+$ cells per mm basement membrane (range 14 – 56) (Figure 3A), whereas in the lamina propria 342 HLA-DR$^+$ cells per mm^2

(range 172 – 424) (Figure 3B) were enumerated. Interestingly, intraepithelial HLA-DR$^+$ cells frequently extended cell projections through the epithelium into the luminal side (Figure 4A).

To further characterize the APC populations we determined the number of CD68$^+$ macrophages and CD11c$^+$ putative DCs (Figures 4B and C). The number of CD68$^+$ and CD11c$^+$ cells were quite similar both in the epithelium and in the lamina propria and the sum of these cell populations was close to 100 % of all HLA-DR$^+$ cells in lamina propria and 60-70% of all HLA-DR$^+$ cells in the epithelial compartment (Figure 3A and B). Additional co-staining experiments showed that there was only 10-20 % overlap between the two markers (Figure 4C), indicating that the mucosal APC populations were mainly made up of CD68$^+$ macrophages and CD11c$^+$ DCs. We and others have found that some AMDCs express CD1a, a marker used to identify epidermal Langerhans cells. Only low densities of CD1a$^+$ cells were found both in the epithelium (median 0.07, range 0 – 1) (Figure 3A) and in lamina propria (median 0.0, range 0.0 – 6) (Figure 3B). pDCs were also found scattered in the lamina propria (median 7, range 2 – 24) and within the epithelium in some specimens (median 0.25, range 0 – 2) (Figure 3A and B)

T- and B cells outside organized lymphoid tissue

CD3$^+$ T cells were observed evenly distributed throughout the surface epithelium and lamina propria (Figure 5). However, the CD45RA$^+$ phenotype was restricted to ILFs demonstrating that mucosal T cells were CD45RA$^-$ memory/effector cells. Only very few CD20$^+$ B cells were found outside ILFs (Figure 5).

DISCUSSION

Here we show that DCs, macrophages and T cells constituted a dense network of immune cells both beneath and within the surface epithelium in the normal bronchial mucosa of children between 1 and 16 years of age. Importantly, organized lymphoid tissue in close proximity to the surface epithelium, identified as BALT, also appeared to be a normal constituent of the local immune system. This shows that the density and composition of immune cells in the lamina propria of the bronchial mucosa in children are very similar to adults. However, in addition the mucosa in children also contains densely dispersed inductive sites which most likely contribute significantly in generating immune responses to inhaled antigens.

We have previously determined the density of APC subsets in bronchial biopsies from 45 infants less than two years of age with respiratory symptoms (18). Both the anatomical distribution and density of HLA-DR$^+$ cells and CD68$^+$ cells were similar to what were reported here. Since there were no signs of inflammation, assessed by infiltration of granulocytes, in any of these studies, the similar results suggest that they represent the homeostatic distribution of APCs in children. Here we also showed that the vast majority of CD11c$^+$ cells were negative for CD68. This finding strengthens the notion that these cells indeed are mucosal myeloid DCs. The individuals in the present study were older than in our previous biopsy study. In the post mortem material there was no correlation between cell numbers and age, like we found in infants from 0-2 years, suggesting that the number of myeloid DCs and macrophages reach steady-state levels at approximately two years of age.

DCs in the gut have been shown to express tight junction proteins and sample bacterial antigen from the gut lumen in response to inflammatory stimuli (19). Our observation that “snorkeling DCs” may be found even in the absence of inflammatory conditions, suggests that this is a phenomenon taking place under homeostatic conditions, and possibly a means by which DCs sample innocuous material for the induction of tolerance (1).

ILFs as part of BALT were observed in mucosal samples from all individuals. They contained naïve T cells, B cells, both CD11c$^+$ myeloid DCs and pDCs, and in some follicles HEVs and follicular DCs were also observed. We estimated the density of ILFs to approximately 60 per square cm demonstrating that these structures are an integral part of the mucosal immune system. In the previous biopsy study (18) we identified ILFs in approximately 50 % of the biopsy samples. Because biopsy specimens from the bronchial

mucosa of infants were very small we would not expect to find ILFs in more than half of the cases with a density of 60/cm^2. Therefore, it seems that BALT develops within months after birth and is an essential component of the mucosal immune system in the bronchi during childhood. Tschernig *et al.* found BALT in children but only in a fraction (44 %) of those who died from trauma (20). The difference between the latter report and ours could at least in part be due to the criteria used to define these structures. We identified ILFs as aggregates of naïve T cell and B cells that were in contact with the overlying surface epithelium and showed that these structures also contained DCs and HEVs. Tschernig et al. based their identification of BALT on routinely stained sections. In our experience, identification of BALT by immunostaining is a much more sensitive technique. Most functional immunological studies have emphasized the importance of DCs migrating to the draining lymph nodes to prime the adaptive immune system. Inducible BALT in mice was recently found to be dependent on CD11c$^+$ DCs for maintenance (21). Here we show that naïve T and B cells are found together with DCs as organized lymphoid tissue beneath and with the surface epithelium in high densities. This may suggest that the immune response to aereoantigens in infants may be initiated to a large extent locally without the participation of the regional lymph nodes. Studies in experimental mice have shown that induced BALT generated strong primary T- and B-cell immune responses when infected with virus (16; 21). These structures should therefore be taken into account when studying the airway immune system in infants and its importance for health and disease.

pDCs have been shown to be vital inducers of tolerance to airway allergens in mouse models (12) and levels of pDCs during infancy were inversely correlated with symptoms of lower respiratory tract infections, parent-reported wheezing, and the cumulative rate of physician-diagnosed asthma up to age 5 years (13). Here we confirm our previous findings that in airway mucosa of children pDCs are primarily located within BALT, but in addition we show that they are also found scattered in the LP and even within the epithelium, suggesting that they may play a role in mucosal homeostasis.

Studies have shown that the immune system in children is immature which explain the high degree of viral infections and allergic sensitization (3). To understand the function of the immune system all structural features must be taken into account. This paper contributes to the understanding of the mucosal immune system in early life. APCs are strategically positioned within the epithelium and actively penetrate the epithelial barrier with cellular extensions making these cells very suitable for sensing the luminal content of antigens. Moreover, aeroantigens may be handled locally by the high number of BALT,

which includes mDCs and pDCs, cells essential in modulating adaptive immune responses. These aspects must be taken into account during mucosal vaccine development.

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FIGURE LEGENDS

Figure 1. The bronchial muocosa of children harbors distinct subepithelial cellular aggregates in which APCs, naïve T cells and B cells accumulate. Immunoenzyme staining for HLA-DR (A and B), CD3 and CD20 (B and C), CD45RA (E and F), CD11c (G), CD123 (H) and MECA (I) of sections from the bronchial wall. HLA-DR$^+$ APCs are diffusely distributed throughout the lamina propria and the epithelium and additionally accumulate in distinct subepithelial aggregates (A and B). These aggregates contain T and B cells, as determined by the expression of CD3 and CD20 (C and D). CD45RA$^+$ naïve T cells accumulate in these aggregates but are not found elsewhere in the mucosa (E and F). CD11c$^+$ mDCs are found at high densities in the aggregates (G). CD123$^+$ pDCs were rarer but found at highest densities within lymphoid aggregates (H). Some lymphoid aggregates contained MECA$^+$ cells, a marker of high endothelial venules (I). Magnifications: (C and E): x40 (A): x100, (D and I): x200, (B and H): x400, (F and G): x600.

Figure 2. BALT was found in all individuals studied. The total numbers of isolated lymphoid follicles (ILFs) were counted in sections stained for CD45RA and CD3/CD20 and given as numbers of ILFs per mm basement membrane
Data are shown with median and interquartile range.

Figure 3. Density of APC subsets in epithelium and in lamina propria. The density of HLA-DR$^+$, CD68$^+$, CD11c$^+$, CD1a$^+$ and CD123$^+$ cells in the epithelium (A) and in lamina propria (B) were calculated per millimeter basement membrane and per mm^2, respectively.

Figure 4. HLA-DR$^+$ and CD11c$^+$ cells in the epithelium extend dendritic projections reaching the luminal surface. Immunoenzyme staining for HLA-DR (A) CD11c (B) and double staining with CD11c and CD68 (C) of sections from bronchial wall. HLA-DR$^+$ cells were seen with extensions reaching the luminal surface (A). Intraepithelial CD11c$^+$ cells also had long extensions, but were not found to reach the surface (B). Double staining with macrophage marker CD68 and DC marker CD11c showed rare double positive cells, approximately 10 % of total CD11c$^+$ numbers. Magnification: all panels x600.

Figure 5. T cells were found throughout the mucosa whereas only very few B cells were observed outside ILFs. Immunoenzyme staining for T cells (CD3) and B cells (CD20) of section from bronchial wall. Magnification: x600.

Figure 1

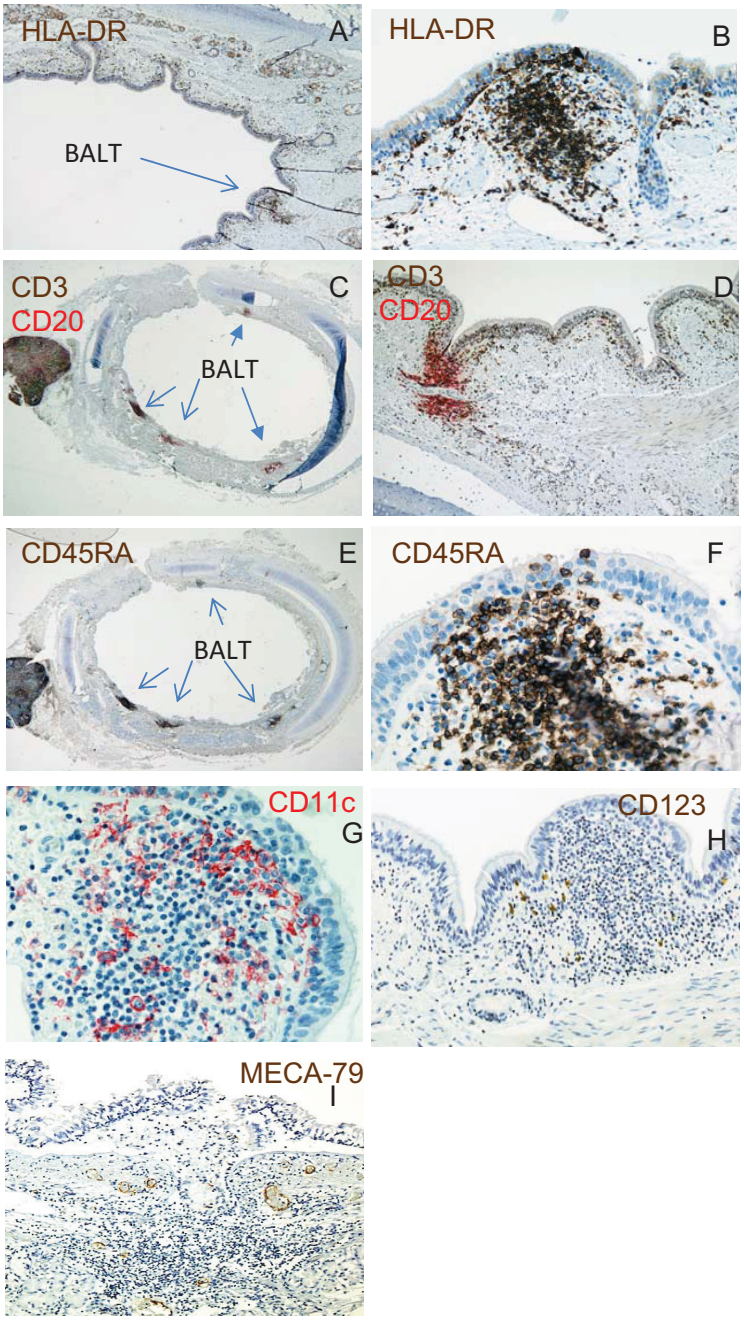
HLA-DR
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Figure 2

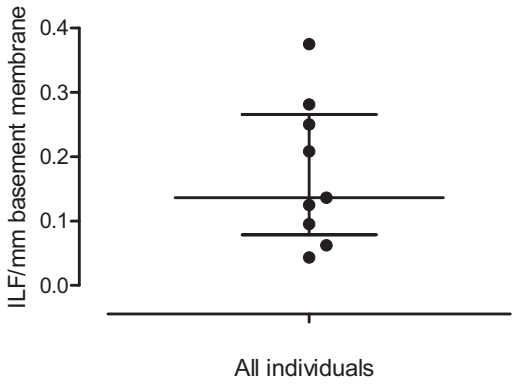


Figure 3

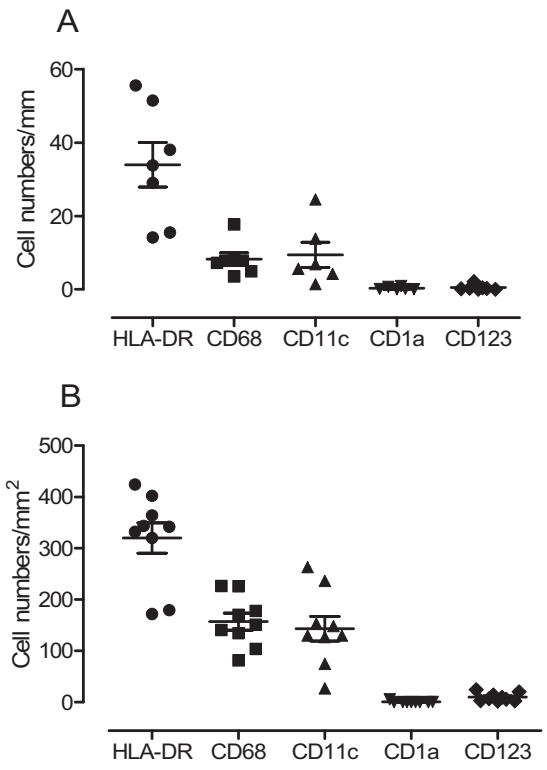


Figure 4

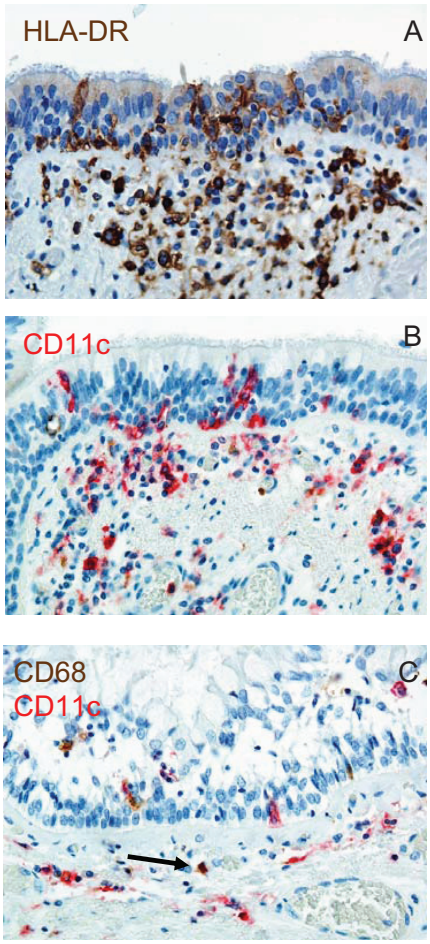
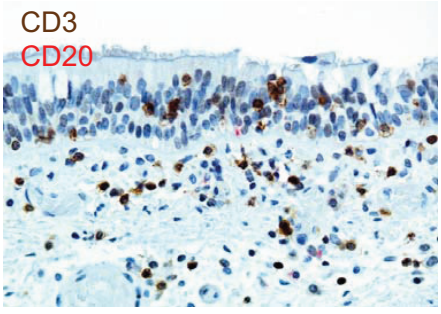


Figure 5



Paper III

SUN EXPOSURE INDUCES RAPID IMMUNOLOGICAL CHANGES IN SKIN AND PERIPHERAL BLOOD IN PSORIASIS PATIENTS.

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III

Sun Exposure Induces Rapid Immunological Changes in Skin and Peripheral Blood in Psoriasis Patients

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ABSTRACT

Background: Sun exposure is a well-described treatment modality for psoriasis and UV-radiation modifies several aspects of the immune system.

Objectives: We wanted to characterise early sun-induced immunological changes both locally and systemically in psoriatic patients.

Methods: 20 patients with moderate to severe psoriasis were subjected to controlled sun exposure on Gran Canaria. PASI-scores were evaluated clinically. Skin biopsies were obtained from lesional and non-lesional skin in ten patients at baseline and day 16 of sun exposure and from five additionally at day 2. Specimens were examined by immunohistochemistry and PCR. Blood samples were obtained from all patients at the same time points and examined for T-cell subsets and cytokine production.

Results: Significant clinical improvement was achieved during the study period. After only one day intraepithelial T cells were markedly decreased and after 16 days $CD4^+$ and $CD8^+$ T cells in lesional skin were significantly reduced in both epidermis and dermis. In contrast, $FOXP3^+$ T cells remained unchanged. We found evidence of reduced mRNA expression of interleukin (IL)-23 and IL-17 in lesional skin. In peripheral blood, skin homing cutaneous lymphocyte-associated antigen $(CLA)^+$ T cells decreased significantly after only one day in the sun and *in vitro*-stimulated peripheral blood mononuclear cells demonstrated reduced capacity to release pro-inflammatory cytokines after 16 days.

Conclusions: Our data show that clinical improvement of psoriasis following sun exposure is preceded by rapid reduction in inflammatory parameters locally and systemically, strongly suggesting that immune modulation mediated the observed effect. We cannot completely rule out that other mechanisms, such as stress reduction, are of any importance. However, it has been extensively documented that UV exposure is a potent inducer of immunosuppression and we therefore find it most likely that the observed effect was primarily due to sun exposure.

INTRODUCTION

Psoriasis is one of the most prevalent autoimmune skin diseases affecting 2-3% of caucasians [1]. It is a chronic disorder with serious medical consequences and impairment of quality of life for patients [2]. Psoriatic skin lesions are sharply demarcated, erythematous, raised, scaling plaques of varying extent but nails, joints and other organs can also be affected [1]. The primary event is widely held to be immunological with hyperstimulatory antigen presenting cells (APCs) and autoreactive T cells in the dermis initiating the pathogenic process [3;4]. The developing cytokine network attracts additional immune cells and perpetuates the inflammatory cascade, inducing the pathological findings characteristic of the disease [5].

Tumor necrosis factor (TNF)-α has been shown to be important in driving immune pathology in psoriasis and the use of anti-TNF-agents is now a well established treatment modality for this disease [6]. However, more recently, the focus of attention has shifted to interleukin (IL)-17-producing T helper cells (Th17), which have been found to be central to the pathogenesis of this disorder [7-10]. IL-23, produced by dendritic cells (DCs) and overexpressed in psoriatic lesions [11], stimulates survival and proliferation of Th17 cells and thus may serve as a key master cytokine regulator for this disease [12]. The therapeutic efficacy of an anti-IL-12/IL-23 antibody on moderate-to-severe plaque psoriasis as well as on psoriasis arthritis was recently demonstrated [13;14].

While therapeutic monoclonal antibodies are effective, their use is expensive and long-term data on safety are still lacking [15]. Natural sun, which has been used for decades in the treatment of psoriasis, has a potent clinical effect potentially involving immunoregulatory mechanisms [16-18]. Immunosuppression following UVB exposure has been described locally in the skin and in the systemic compartment [19]. Induction of regulatory T cells (Treg) has been suggested to be one mechanism mediating the clinical effect of UVB treatment [20;21]. Vitamin D, produced locally in the skin upon sun exposure, has also been shown to contribute to the immunological changes seen in the skin upon UVB exposure [21;22]. However, most of these studies are performed in mouse models and human data are scarce. On this background, we wanted to study the early immunomodulatory effects of sun exposure in psoriatic patients both *in situ*, in non-lesional and lesional skin, as well as in the systemic compartment.

MATERIALS AND METHODS

Subjects

Heliotherapy is a well established therapeutic modality for patients with psoriasis in Scandinavian countries and approximately 500 Norwegian psoriatic patients are selected for heliotherapy annually.

20 patients (median age 48 years, range 24-65, 6 females) were enrolled in the study and transported from Norway to Gran Canaria, Spain, in the month of March. The patients were evaluated by the same dermatologist for the Psoriasis Area and Severity Index (PASI) [23] before and after sun treatment. All patients had moderate to severe plaque psoriasis, i.e. mean/median PASI before climatotherapy of 9.8/8.7, range 3.8-18.8. All patients had stopped using any psoriasis medication at least 4 weeks prior to this study. Two of the patients had skin type II and 18 had skin type III according to the Fitzpatrick classification [24]. Patients eligible to receive climate therapy are preferred to have darker skin types in order to avoid potentially adverse effects of sun exposure. UV-doses were measured and details were described by Nilsen et al. [25].

The study followed the protocols of the Helsinki declaration and was approved by the Regional Committee of Medical Ethics. All patients gave their written, informed consent.

Skin biopsies and blood samples

4 mm punch biopsy samples were collected from lesional and non-lesional skin in 10 randomly selected patients. The samples from each individual patient were obtained within the same body area on all days but in sufficient distance to avoid a reactive inflammation from prior biopsy sampling. Specimens from 5 patients at obtained baseline and at day 16 were formalin-fixed and paraffin-embedded, whereas samples from another 5 patients obtained at baseline and days 2 and 16 were snap frozen in liquid nitrogen and stored at -80˚ C until sectioning.

Venous blood samples were collected from all 20 patients on days 0, 2 and 16. Mononuclear cells were isolated immediately for further investigations (see below).

Immunohistochemistry

Formalin-fixed and paraffin-embedded biopsies were cut at 4 μm. Immunoenzyme staining for CD1a (mouse IgG1, clone MTB1, Novocastra, Newcastle, UK) and CD8 (mouse IgG1,

clone C8/144B, DakoCytomation, Glostrup, Denmark) on dewaxed tissue sections was performed in a Ventana NexEs IHC instrument (Tucson, AZ) with the standardized iView DAB detection kits as recommended by the manufacturer. Two-colour immunofluorescence staining of formalin-fixed specimens was performed combining rabbit anti-CD3 (clone SP7, Thermo Fisher Scientific, Fremont, CA), and mouse anti-CD4 (IgG1, clone 1F6, Novocastra) or rabbit polyclonal anti-CD3 (DakoCytomation) and mouse anti-FOXP3 (IgG1, clone 259D/7C, BD Pharmingen, San Diego, CA). The following secondary antibodies were used: Alexa 555 goat anti-rabbit, Alexa 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit IgG (all from Molecular Probes, Eugene, OR) and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA).

Cryosections were cut 4 μm thick and fixed in acetone prior to staining. Two-colour immunofluorescence staining was performed combining rabbit polyclonal anti-CD3 (DakoCytomation) with mouse anti-CD4 (IgG1, clone SK3, BD Pharmingen), mouse anti-CD8 (IgG1, clone SK1, BD Pharmingen) or mouse anti-FOXP3 (clone 259D/7C, BD Pharmingen). Staining for LCs was performed with anti-CD1a (clone NA1/34, DakoCytomation). The following fluorescence-labelled secondary antibodies were used: Alexa 488 goat anti-rabbit IgG (Molecular Probes) and Cy3 goat anti-mouse IgG (Jackson Immunoresearch).

Stained sections were examined by light or fluorescence microscopy at x 400 magnification by the same investigator (IH). Cell numbers were recorded using an ocular grid (250μm x 250μm), counting positive cell profiles in epidermis and in the papillary and reticular dermis, to a depth of 250 μm. For the grossly thickened epidermis of psoriatic lesions, cell numbers per square millimetre rather than per millimetre of surface epidermis will reflect the cell density more accurately. Data for both dermal and epidermal cell counts are given as cells numbers per square millimetre, in both lesional and non-lesional skin, in order to make comparisons.

mRNA from skin biopsies

RNA was isolated from 5 x 14 μm cryosections collected in 1.5 ml eppendorf tube containing 500 μl TRI Reagent Solution (Applied Biosystems; Foster City, CA) and 1 μg used for a 20-μl cDNA synthesis reaction with SuperScript III (Invitrogen, Carlsbad, CA) and 20 pmol oligo dT. Primers for real-time PCR were designed with primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences are given in Table 1. 1 μl cDNA was used as template for real-time PCR in Stratagene MX3000P with 0.125

U/µl HotStar Taq polymerase (Qiagen, Hilden, Germany), EvaGreen® (Biotium; 0.5x recommended amount) and $MgCl_2$ concentration as indicated in Table 1. PCR conditions included 15 min 95ºC followed by cycling conditions as follows: 95ºC, 30 sek; annealing 60ºC 30 sek; 72 ºC 30 sek. Each target mRNA was quantified by Ct (threshold crossing point) values using a cDNA pooled from several IBD patients to generate a standard curve and related to the GAPDH mRNA level. Melting curves were monitored for specificity of PCR products.

Flow cytometric analysis of peripheral blood T cells

EDTA-whole blood was incubated with monoclonal antibodies in the following combinations: CD3 peridinin chlorophyll protein (PerCP)/CD4 phycoerythrin (PE)/CLA-fluorescein isothiocyanate (FITC)/CD45 allophycocyanin (APC) and CD3-PerCP/CD8-PE/CLA-FITC/CD45-APC, and with isotypic-matched irrelevant antibodies (all from BD Biosciences, San Jose, CA). After 20 minutes at room temperature in the dark, erythrocyte lysis was performed. Samples were then washed in PBS and cells were examined by flow cytometry (FACSCalibur®, BD Biosciences). At least 10.000 lymphocytes were gated by forward and side scatter and CD45 expression. The collected data were analyzed using CellQuest Pro software (Apple computer, Inc; Cupertino, CA).

Release of cytokines from PBMCs

PBMCs, obtained from heparinized blood by isopaque-Ficoll (Lymphoprep, Nycomed Pharma, Oslo, Norway) gradient centrifugation, were incubated in flat-bottomed 96-well trays (Costar, Corning Inc., Corning, NY; $2x10^6$ cells/mL; 100 µL/well) in medium alone (RPMI-1640 containing 2 mM L-glutamine [Sigma Chemical Co., St. Louis, MO], supplemented with 100 U/mL penicillin and 5% fetal calf serum), or with phytohaemagglutinin (PHA; Murex, Dartford, UK; final concentration 5 µg/mL). Cell-free supernatants were harvested after culturing for 24 hours and stored at -80°C until analysis.

Cytokine measurements

Cytokine levels in cell-free supernatants were analysed using a multiplex cytokine assay (Bio-Plex Human Cytokine 8-Plex Panel, Bio-Rad Laboratories Inc., Hercules, CA), according to the instructions from the manufacturer.

Statistical analysis

Paired non-parametric tests Wilcoxon signed rank test was used to examine differences within the same individuals over time. Because biopsy specimens from day 2 were available from only 5 individuals, these *in situ*-data were omitted from the statistical analyses. *P*-values <0.05 were interpreted as significant

RESULTS

Clinical score

All patients experienced clinical improvement, and the mean reduction of Psoriasis Area and Severity Index (PASI) scores was 72.8% after 16 days of climatotherapy (Fig. 1a).

16 days of sun exposure induced reduction in lesional epidermal thickness

Biopsies obtained from lesional and non-lesional skin at baseline at day 2 and 16 of sun exposure were examined by immunohistochemistry. At baseline, non-lesional skin was normal histologically, whereas lesional skin showed severe pathology with parakeratosis, thickened epithelium, elongated papillae and inflammatory infiltrates (Fig. 2a-b). No changes in epidermal thickness were seen after sun exposure in non-lesional skin (Fig.1b). In lesional skin 16 days of sun exposure induced a significant reduction in epidermal thickness (Figs.1b and 2b-c).

T cell populations in the epidermal and dermal compartments are rapidly reduced by sun exposure

As T cells are central in driving psoriasis pathology [3;4], we wanted to examine the early impact of sun exposure on $CD4^+$, $CD8^+$ and regulatory T-cell populations *in situ*. Samples (frozen or formalin-fixed) from 10 patients were examined by immunohistochemistry and positive stained cells were enumerated. Cell densities are presented as cell numbers per mm^2 for the epidermal (Fig. 3) and dermal (Fig. 4) compartments separately.

Epidermal compartment

Sections from non-lesional skin contained low numbers of epidermal T cells (Figs. 2, left panels and 3a, c and e). $CD4^+$ T cells infiltrating the epidermis were moderately elevated in lesional skin, (P=0.0005, Figs. 2e and 3b), whereas epidermal $CD8^+$ T cells were

dramatically increased (P<0.0001, Figs. 2h and 3d). Sun exposure resulted in significant reductions in epidermal $CD4^+$ T cells in both non-lesional and lesional skin (Figs. 2f and 3a-b). Epidermal $CD8^+$ T cells in non-lesional skin were unaffected (Fig. 3c), whereas in lesional skin, they were reduced to non-lesional levels at day 16 (Figs. 2h-i and 3d). Both $CD4^+$ and $CD8^+$ T cells were markedly reduced in the epidermis already at day 2, although these differences could not be tested statistically (Fig. 3b and d). $FOXP3^+$ T cells (putative Tregs) were increased in lesional epidermis compared with non-lesional (P=0.033). Non-lesional epidermal $FOXP3^+$ T cells were unaffected by sun exposure (Fig. 3e), whereas in lesional skin, they were significantly reduced at day 16 (Figs. 2k-l and 3f).

Dermal compartment

$CD4^+$ and $CD8^+$ T cells were significantly increased at in lesional dermis at baseline compared with non-lesional dermis (P<0.0001 for both subsets, Fig. 4a-d). In non-lesional dermis, no significant changes in $CD4^+$ or $CD8^+$ T cell numbers were observed after sun exposure (Fig. 4a and c). In lesional dermis, however, both T-cell subsets were significantly reduced on day 16 of sun exposure (Fig. 4b and d).

Dermal $FOXP3^+$ T cells were also increased in lesional skin (P=0.002, Fig. 4e-f). In lesional dermis, the number of $FOXP3^+$ T cells remained unchanged after sun exposure (Fig. 4f), suggesting a relative increase in Tregs, although the percentage of $FOXP3^+$ $CD4^+$ T cells did not reach statistical significance (data not shown).

For all T cell subsets studied, a redistribution of infiltrates could be observed after sun exposure, with the most dramatic reduction in cell numbers in the epidermis and papillary dermis, whereas infiltrates in the reticular dermis persisted (Fig.2).

The density of Langerhans cells was decreased in lesional epidermis

In contrast to the other cell populations studied, the densities of Langerhans cells (LCs), defined as epidermal $CD1a^+$ cells per mm^2, were profoundly reduced in lesional skin at baseline, compared with non-lesional epidermis (P<0.0001, Figs. 3g-h and 5a and c). In non-lesional epidermis LCs, as expected, were dramatically reduced upon sun exposure (Figs. 3g and 5a-b). In lesional epidermis, there was a slight but significant reduction in LC numbers after sun exposure (Figs. 3h and 5c-d). There was no difference in numbers of dermal $CD1a^+$ cells between non-lesional and lesional skin at baseline. The density of dermal $CD1a^+$ cells remained unchanged in non-lesional dermis after sun exposure, whereas in lesional dermis a significant reduction was observed (Figs. 4g-h and 5a-d).

Cytokine gene expression in lesional skin

In order to investigate whether sun induced reduction in T cell numbers was associated with reduction in cytokine gene expression mRNA was isolated from the same frozen biopsies that were examined by immunohistochemistry. In samples from non-lesional skin, very low levels of cytokines were detected (data not shown). From lesional specimens, unfortunately, only samples from four individuals yielded results of satisfactory quality, so no statistics could be performed on these data. However, clear trends could be observed for some of the cytokines. Expression levels of TNF-α was markedly reduced in 3 out of 4 individuals at day 2, whereas it was unchanged at day 16 (Fig. 6a). IL-12p40 (also part of IL-23) was reduced in 4 out of 4 individuals at day 16 (Fig. 6b). IL-23p19 and IL-17, showed markedly reduced expression levels in 3 out of 4 individuals on both day 2 and day 16 of sun exposure (Fig. 6c-d). In contrast, expression levels of the anti-inflammatory cytokine IL-10 was increased in 3 out of 4 individuals on day 16 (Fig. 6e), whereas transforming growth factor (TGF)-β seemed unchanged (Fig. 6f).

The frequency of CLA^+T cells in peripheral blood was reduced already at day 2 of sun exposure

To study the impact of immune function in the systemic compartment, we next examined whether sun exposure affected circulating skin homing T cells, characterized by expression of the homing marker cutaneous lymphocyte antigen (CLA). PBMCs obtained from blood samples from all 20 patients at baseline, day 2 and day 16 of sun exposure, were examined by flow cytometry. Total numbers of $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells were unaffected by sun exposure (data not shown). The frequencies of both $CD4^+$ and $CD8^+$ CLA^+ T cells as a percentage of total T cells (not shown) as well as a percentage of $CD4^+$ and $CD8^+$ T cells (Fig. 7a-b) were significantly decreased already at day 2 and remained low at day 16 of sun exposure, demonstrating a selective reduction of CLA^+ T cells in the peripheral blood.

Release of cytokines from peripheral blood mononuclear cells (PBMCs) *ex vivo*

We next examined the release of cytokines from unstimulated and PHA-stimulated PBMCs obtained from all 20 patients at baseline, day 2 and day 16. Supernatants were removed after culturing cells for 24 hours. In supernatants from unstimulated cells, all cytokine concentrations were below detection limit (data not shown). There was no difference in cytokine secretion in PBMCs obtained after 2 days of sun exposure. After16 days, PBMCs

released significantly less interferon (IFN)-γ, IL-17, TNF-α and IL-10 compared to baseline levels (Fig. 8a-d), whereas the reduction in IL-12p40 release was non-significant (Fig. 8e).

DISCUSSION

This study demonstrated that 16 days of natural sun exposure induced excellent clinical improvement in psoriatic patients. The clinical response was preceded by rapid immunological changes both *in situ* and systemically.

Previous studies have demonstrated that UVB irradiation induces apoptosis of T cells but not $CD1a^+$ cells in psoriatic lesions [26;27]. The fact that the most pronounced depletion of both $CD4^+$ and $CD8^+$ T cells was seen in the epidermis and upper parts of the papillary dermis, strongly suggests that apoptosis could contribute to the massive reduction of T cells upon sun exposure in affected skin in our study population [27]. In order to examine the specimens for evidence of apoptosis, we stained for cleaved caspase-3, but could not detect any positive signals, in spite of good results in positive control samples. We speculate that one explanation for this could be due to timing and that the product of apoptosis detected by this method may not be optimally expressed at the time points on which our biopsies were sampled.

Epidermal $FOXP3^+$ cells were near depleted along with the two main cell subsets. In contrast, dermal $FOXP3^+$ T cells remained unchanged after 16 days of sun exposure, suggesting that the induction of Tregs is one of the mechanisms mediating sun induced improvement in psoriasis. A possible explanation for the differential effect on $FOXP3^+$ T-cell numbers in the two compartments could be that the dermal cells are less susceptible to apoptosis and that newly recruited $T_{regs,}$ reflecting a change in the local cytokine milieu, will be found in the dermis under such circumstances.

Unfortunately, our mRNA data were of insufficient quality to enable us to draw conclusions, but they suggest that reductions in cell numbers are paralleled by reduction in pro-inflammatory cytokines. It has previously been shown that 3 weeks of UVB treatment reduced expression of IL-23p19 [28] and that 8 weeks of cyclosporine treatment reduced IL-17 expression in psoriatic skin [29]. Together, these data are in line with recent reports [9;30;31] demonstrating that Th17 cells are central in immunopathology of psoriasis and indicate that sun exposure may down-regulate the IL-23/IL-17-axis, potentially contributing to its beneficial effects in psoriatic patients. The increased IL-10 mRNA levels in lesional skin in

3 out of 4 individuals at day 16 also supports the concept of a change from an inflammatory into a more homeostatic environment upon sun exposure.

We demonstrate for the first time that skin homing CLA^+CD4^+ and CLA^+CD8^+ T cells in peripheral blood decreases significantly in psoriatic patients after only one day of sun exposure. Previously, it has been shown that UVB exposure reduces total CLA^+ T cells in the blood of psoriatic patients after one week [32]. The rapid decrease of CLA^+ T cells in peripheral blood could be caused by an increased migration to both lesional and non-lesional skin. However, cell numbers in non-lesional skin were unchanged whereas they were markedly reduced in lesional skin after sun exposure. As CLA^+ T cells preferentially circulate to the skin, they would be susceptible to apoptosis and thus peripheral numbers would decrease. Whether UV-irradiation could have a direct down-regulatory effect on CLA has not been established. The attenuated capacity of *in vitro*-cultured PBMCs to produce cytokines after sun exposure, suggests that sun exposure also induced functional changes in the systemic compartment. This is in line with previous studies on the immunomodulatory effects of UV radiation and could be mediated by several different mechanisms [33;34].

LCs have previously been found in decreased densities in psoriatic lesions [35]. We confirm this finding and our data also support previous reports of LCs having impaired migratory function in psoriatic patients [36]. LCs normally leave the epidermis and migrate to regional lymph nodes upon UV-exposure [37]. However, we found that LCs in lesional epidermis, in contrast to $CD4^+$ and $CD8^+$ and $FOXP3^+$ lymphocyte populations, were only slightly reduced in numbers upon sun exposure, suggesting impaired migratory function. LCs in non-lesional epidermis, as expected, were dramatically reduced after sun exposure, suggesting that this impaired migration is not a primary defect in psoriasis.

Warm climate and bathing in combination with sun exposure might reduce stress and thereby indirectly improve the psoriasis lesions in addition to the UV-induced effects. However, there is a large body of evidence to suggest that UV exposure under experimental conditions have a very strong immunosuppressive effect, which is compatible with the idea that reduction in pathogenic T cells is mainly due to sun treatment.

In conclusion, the present study shows that exposure to natural sun induces excellent clinical improvement within 16 days associated with a rapid reduction of skin homing T cells from the peripheral blood and of T cells from lesional skin with a relative increase in

Tregs. The concomitant change in the systemic cytokine profile favoring an immunosuppressive environment, may contribute to the resolution of inflammation.

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mRNA target (gene name)	Primer sequence (5' to 3', forward primer written on top)	Product size (bp)	$MgCl_2$ conc. (mM)
hIL10	TTACCTGGAGGAGGTGATGC GGCCTTGCTCTTGTTTTCAC	148	2.0
hIL12B (p40)	TACTCCACATTCCTACTTCT CGTGAAGACTCTATCTTTCT	85	4.0
hIL17A	AGGCACAAACTCATCCATCC GCTCAGCAGCAGTAGCAGTG	91	2.5
hIL23A (p19)	GACACATGGATCTAAGAGAA AGCAGAACTGACTGTTGTC	114	3.0
hTGFB1	GTGGAAACCCACAACGAAAT CGGAGCTCTGATGTGTTGAA	83	2.5
hTNF	AGCCCATGTTGTAGCAAACC TGAGGTACAGGCCCTCTGAT	134	2.5
hGAPDH	TGTTCGACAGTCAGCCGCATCTTCT TGATGGCAACAATATCCACTTTACCAGAGTT	161	2.0

Table 1 Primer sequences used for RT- PCR analyses

FIGURE LEGENDS

Figure 1. PASI-scoring and reduction in epidermal thickening. Twenty patients received controlled natural sun exposure for 16 days. All patients showed clinical improvement and PASI-scoring showed a significant reduction at day 16 (a). Epidermal thickness in specimens was unchanged in non-lesional skin after 16 days of sun exposure (n=10), whereas in lesional skin epidermal thickness was significantly reduced (n=10) (b).

Figure 2. Histology and immunohistochemistry of cryosections (a-i) and formalin fixed sections (j-m). Sections shown are from non-lesional skin at baseline (left panels), from lesional skin at baseline (middle panels) and after 16 days of sun exposure (right panels). Hematoxyline-Eosin-stained sections showed a reduction in epidermal thickness and of inflammatory cell infiltrates (a-c). In situ phenotypic characterization of T cell populations was performed by paired immunofluorescence staining with CD3 (Alexa 488, green) and CD4 (Cy3, red) (d-f), CD8 (Cy3, red) (g-i), and FOXP3 (Cy3, red) (j-m). Double positive cells appear yellow. The white dotted line denotes the dermo-epidermal junction. Note the reduction in T cells populations in lesional skin at day 16, especially in epidermal and papillary dermal compartments. Images (a-l) are at 200x magnification. Panel (m) shows $CD3^+FOXP3^+$ cells in lesional dermis at 600x magnification. The anti-FOXP3-antibody showed unspecific staining of the upper epidermis in all specimens obtained after sun exposure (l).

Figure 3. Numbers of immunostained cells expressed per mm^2 for the epidermal compartment in non-lesional (left panels) and lesional skin (right panels) at baseline and on days 2 and 16 of sun exposure (n=10). Numbers for the following cell populations are shown: $CD3^+CD4^+$ cells (a and b), $CD3^+CD8^+$ cells (c and d), $CD3^+FOXP3^+$ cells (e and f) and $CD1a^+$ cells (g and h).

Figure 4. Numbers of dermal immunostained cells expressed per mm^2 in non-lesional (left panels) and lesional skin (right panels) at baseline and on days 2 and 16 of sun exposure (n=10). Numbers for the following cell populations are shown: $CD3^+CD4^+$ cells (a and b), $CD3^+CD8^+$ cells (c and d), $CD3^+FOXP3^+$ cells (e and f) and $CD1a^+$ cells (g and h).

Figure 5. $CD1a^+$ Langerhans cells in non-lesional (upper panels) and lesional skin (lower panels) at baseline (left panels) and at day 16 of sun exposure (right panels). The density of epidermal $CD1a^+$ LCs was reduced in lesional skin compared with non-lesional skin (a and b). After sun exposure, LCs were dramatically reduced in non-lesional epidermis (c), whereas the reduction in lesional epidermis was significant but less pronounced (d).

Figure 6. Cytokine mRNA in frozen biopsy specimens obtained at baseline and on days 2 and 16 after sun exposure. Unfortunately, only 4 specimens yielded qualitatively satisfying results, so no statistics could be performed. RT-PCR was performed to investigate expression levels of the following cytokines, TNF-α (a), IL-12p40 (common subunit of IL-12 and IL-23) (b), IL-23p19 (c), IL-17 (d), IL-10 (e) and TGF-β (f). Expression levels are normalized to GAPDH.

Figure 7. CLA^+ T lymphocyte subpopulations in peripheral blood at baseline and on days 2 and 16 of sun exposure analysed by flow cytometry. The percentage of $CD4^+CLA^+$ cells of $CD4^+$ T cells (a) and $CD8^+CLA^+$ cells of $CD8^+$ T cells (d) were significantly reduced. Data are mean±SEM of all 20 patients. ***, $P<0.001$, **, $P<0.01$ versus baseline.

Figure 8. Cytokine release from PHA-stimulated PBMCs obtained at baseline and on days 2 and 16 of sun exposure. The release of IFN-γ (a), IL-17 (b), TNF-α (c), IL-10 (d) were all significantly reduced in PBMCs obtained at day 16. The reduction in the release of IL-12p40 (common subunit of IL-12 and IL-23) (e) did not quite reach statistical significance. Data are mean±SEM of all 20 patients. **, $P<0.01$; *, $P<0.05$; #, $P=0.064$ versus baseline.

Figure 1

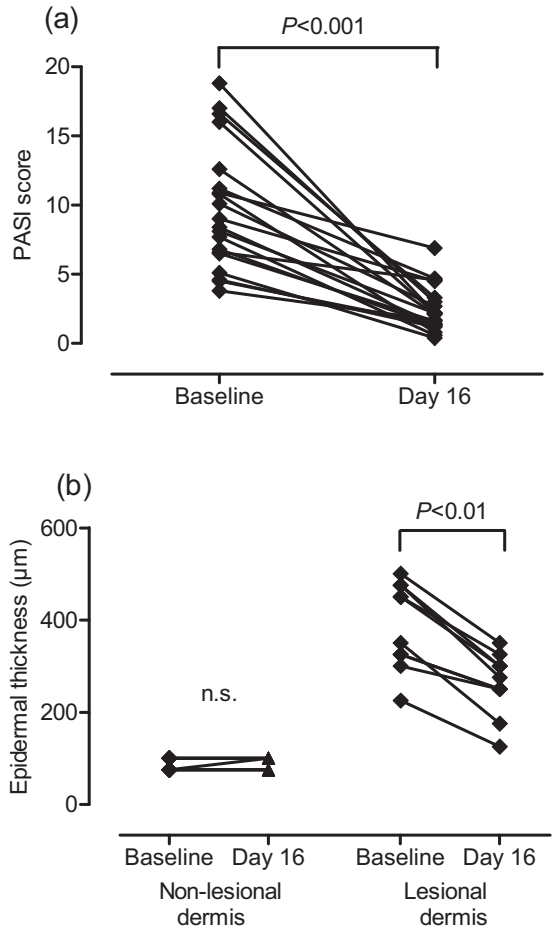

(a)
P<0.001
20
15
10
5
0
PASI score
Baseline
Day 16
(b)
P<0.01
600
400
200
0
Epidermal thickness (μm)
n.s.
Baseline
Day 16
Baseline
Day 16
Non-lesional dermis
Lesional dermis

Figure 2

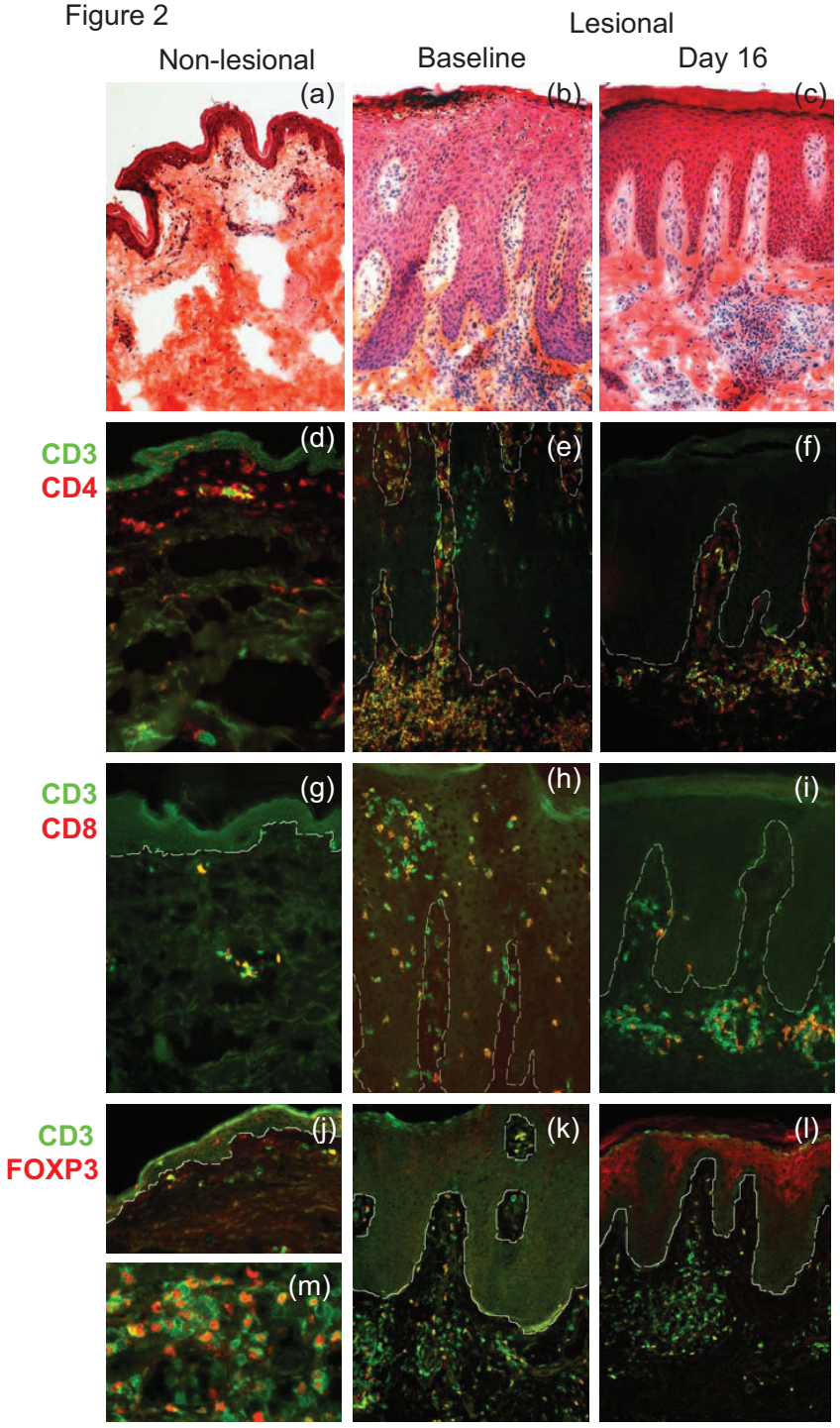

Lesional
Non-lesional
Baseline
Day 16
(a)
(b)
(c)
CD3
CD4
(d)
(e)
(f)
CD3
CD8
(g)
(h)
(i)
CD3
FOXP3
(j)
(k)
(l)
(m)

Figure 3

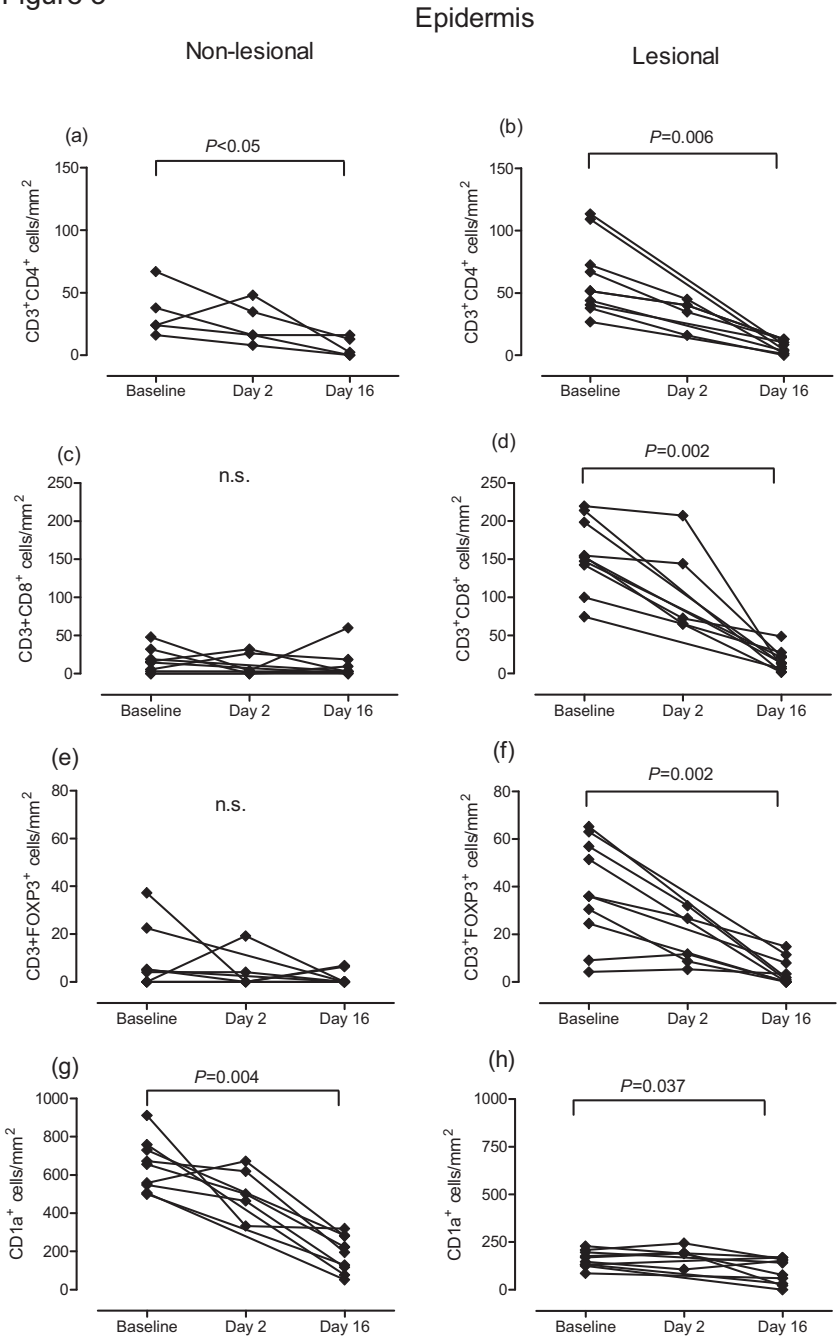

Epidermis
Non-lesional
Lesional
(a)
P<0.05
CD3+CD4+ cells/mm2
(b)
P=0.006
CD3+CD4+ cells/mm2
(c)
n.s.
CD3+CD8+ cells/mm2
(d)
P=0.002
CD3+CD8+ cells/mm2
(e)
n.s.
CD3+FOXP3+ cells/mm2
(f)
P=0.002
CD3+FOXP3+ cells/mm2
(g)
P=0.004
CD1a+ cells/mm2
(h)
P=0.037
CD1a+ cells/mm2
Baseline
Day 2
Day 16

Figure 4

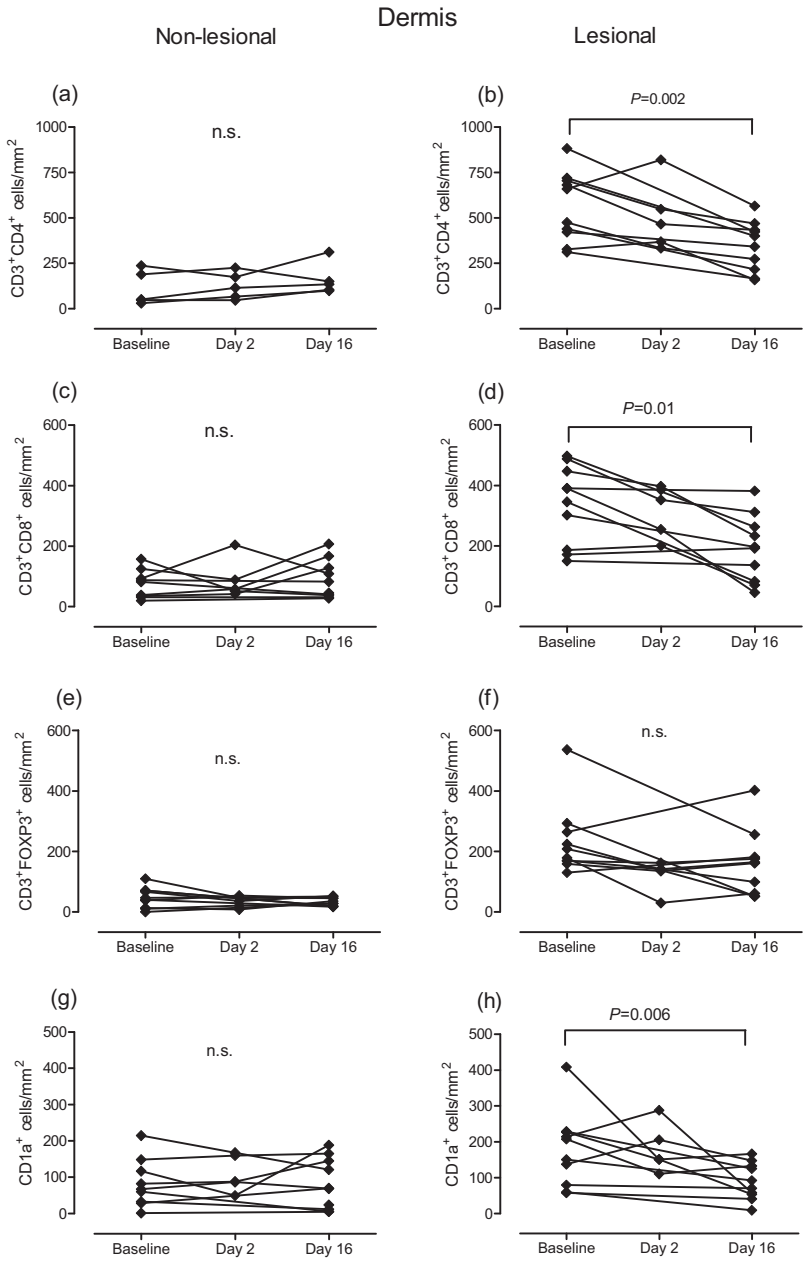

Dermis
Non-lesional
Lesional
(a)
n.s.
CD3+CD4+ cells/mm2
1000
750
500
250
0
Baseline
Day 2
Day 16
(b)
P=0.002
(c)
n.s.
CD3+CD8+ cells/mm2
600
400
200
(d)
P=0.01
(e)
n.s.
CD3+FOXP3+ cells/mm2
(f)
n.s.
(g)
n.s.
CD1a+ cells/mm2
500
300
100
(h)
P=0.006

Figure 5

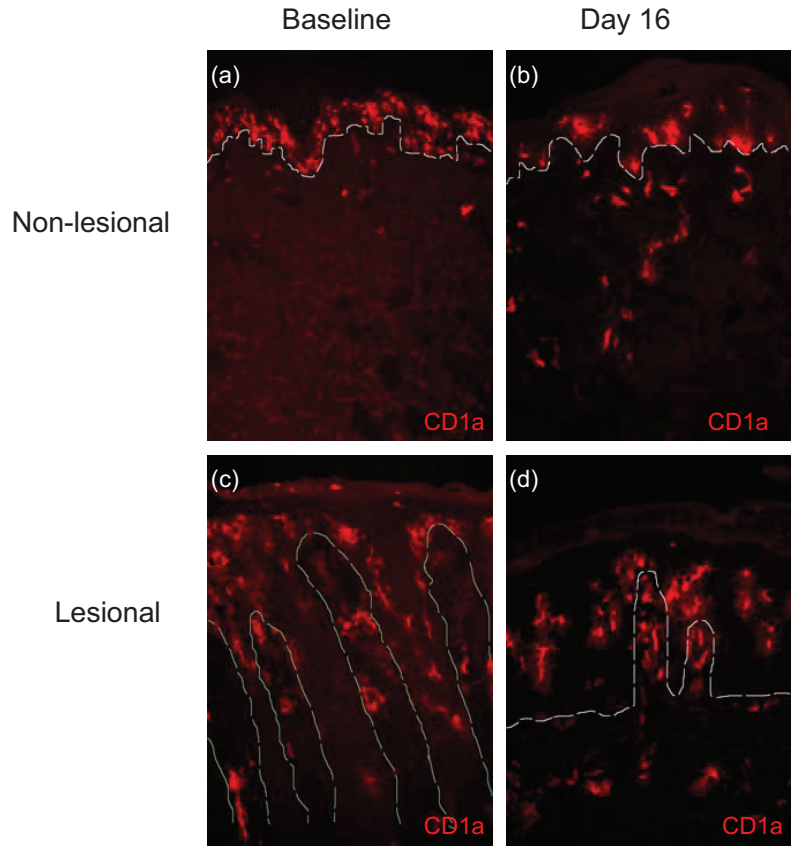

Baseline
Day 16
Non-lesional
Lesional
(a)
(b)
(c)
(d)
CD1a
CD1a
CD1a
CD1a

Figure 6

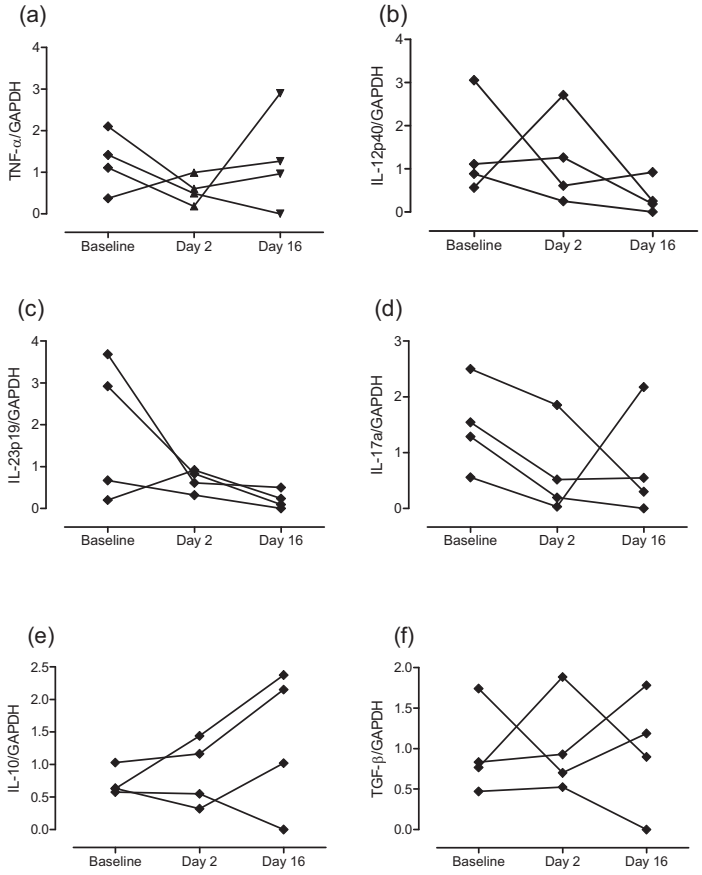


Figure 7

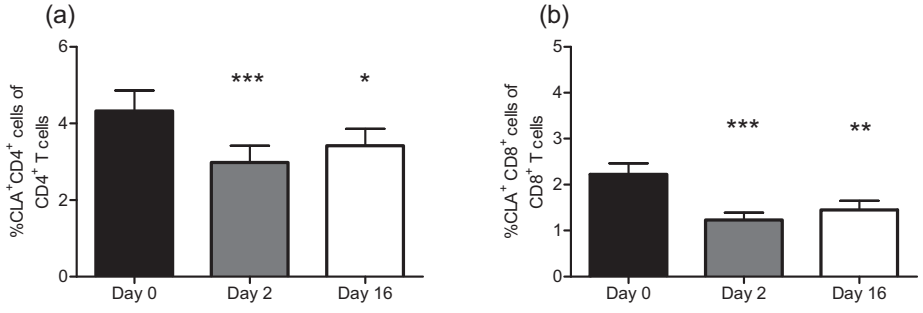
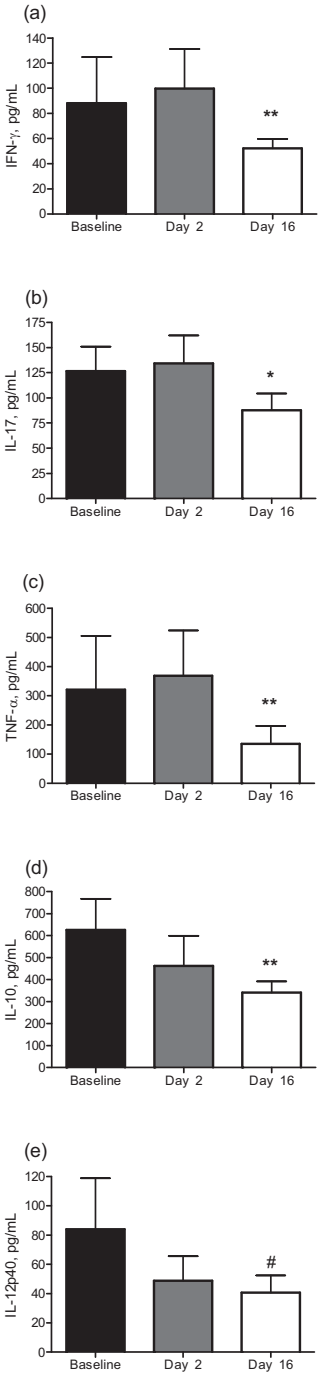


Figure 8


(a)
IFN-γ, pg/mL
140
120
100
80
60
40
20
0
**
Baseline
Day 2
Day 16
(b)
IL-17, pg/mL
175
150
125
100
75
50
25
0
*
Baseline
Day 2
Day 16
(c)
TNF-α, pg/mL
600
500
400
300
200
100
0
**
Baseline
Day 2
Day 16
(d)
IL-10, pg/mL
800
700
600
500
400
300
200
100
0
**
Baseline
Day 2
Day 16
(e)
IL-12p40, pg/mL
120
100
80
60
40
20
0
#
Baseline
Day 2
Day 16

Paper IV

SUN EXPOSURE RAPIDLY REDUCES PLASMACYTOID DENDRITIC CELLS AND INFLAMMATORY DERMAL DENDRITIC CELLS IN PSORIATIC SKIN

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Paper submitted 2009

IV

Sun Exposure Rapidly Reduces Plasmacytoid Dendritic Cells and Inflammatory Dermal Dendritic Cells in Psoriatic Skin

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ABSTRACT

Interferon (IFN)-α producing plasmacytoid dendritic cells (pDCs) and inflammatory $CD11c^+CD1c^-$ myeloid DCs accumulate in the dermis of psoriatic lesions and are believed to play a central role in the pathogenesis of the disease. Here, we show that 16 days of heliotherapy had excellent clinical effect on psoriatic patients that concurred with reduced numbers of pDCs and reduced expression of MxA, a surrogate marker for IFN-α, in lesional skin. A rapid reduction in $CD11c^+CD1c^-$ myeloid DCs (mDCs) was also observed whereas the density of resident dermal $CD11c^+CD1c^+$ mDCs was unaffected. Sun exposure also led to reduced expression of the maturation marker DC-LAMP on mDCs. A significant decrease in lesional dermal macrophages was also observed. In conclusion, we show that the clinical effect of sun exposure in psoriasis is strongly associated with rapid changes in dermal DC populations in lesional skin, which partly precede the clinical effect. These findings add further evidence to the notion that these cells are directly involved in the pathogenesis of the disease, and suggest that UV-induced immunosuppression, at least in part, can be explained by its effect on dermal APCs.

INTRODUCTION

Psoriasis is a chronic autoimmune skin disorder believed to be mediated by pathogenic interactions between dendritic cells (DCs), T cells and keratinocytes (Lowes *et al.*, 2007). Several DC subsets have been shown to accumulate in psoriatic skin and, as efficient antigen presenting cells (APCs), they are thought to stimulate pathogenic T cells locally (Lowes *et al.*, 2007;Nickoloff *et al.*, 2007). This concept is underscored by the fact that several new biologics with clinical effect in psoriasis specifically target molecules involved in DC-T-cell interactions (Tzu and Kerdel, 2008).

Plasmacytoid (p)DCs are present in very low numbers in normal skin (Zaba *et al.*, 2009b) but recent reports have shown that pDC numbers are increased in psoriatic lesions and contribute to immune pathology, mainly through the production of interferon (IFN)-α (Nestle *et al.*, 2005;Yao *et al.*, 2008). Interestingly, a recent publication demonstrated that pDCs are activated through toll-like receptor (TLR) 9 via endogenous DNA coupled with the antimicrobial peptide LL37, which is upregulated in psoriasis (Lande *et al.*, 2007).

Under steady-state conditions human dermal myeloid APCs have been found to consist of functionally and phenotypically distinct populations of myeloid (m)DCs and macrophages (Zaba *et al.*, 2007b). Resident dermal mDCs are characterized by the expression of CD11c and CD1c (Zaba *et al.*, 2009b). Dermal macrophages are usually $CD11c^-$ but express CD163 (Zaba *et al.*, 2007b). Although the C-type lectin DC-specific integrin ICAM-3 grabbing non-integrin (DC-SIGN, CD209) was previously believed to be a specific marker for DCs, it has more recently been found to be expressed primarily on macrophages in normal skin (Ochoa *et al.*, 2008) as well as in other tissues (Raki *et al.*, 2006).

In psoriatic lesions an additional mDC subtype expressing CD11c but not CD1c has been identified (Zaba *et al.*, 2009b). These cells have been termed inflammatory DCs, and a proportion of these, but not the $CD11c^+CD1c^+$ resident subset, also expresses the monocyte/macrophage markers CD163, DC-SIGN and CD14 (Zaba *et al.*, 2009a). Moreover, these inflammatory dermal mDCs were shown to produce TNF-α, inducible nitric oxide synthase (iNOS), interleukin (IL)-23, and to activate both Th1 and Th17 T cells and could therefore potentially be central players in psoriasis pathogenesis (Zaba *et al.*, 2009a).

In addition to pDCs and inflammatory mDCs, macrophages have also been implicated in psoriasis pathogenesis. They were shown to be a source of pathogenic TNF-α in mouse models of the disease (Stratis *et al.*, 2006;Wang *et al.*, 2006) and there is evidence to suggest that macrophages contribute to psoriasis also in humans (Marble *et al.*, 2007;Wang *et al.*, 2009a).

We have recently shown that significant clinical improvement during 16 days of sun exposure was associated with rapid reduction in T-cell numbers in lesional skin as well as downregulation of inflammatory parameters both *in situ* and systemically (Søyland *et al,* manuscript accepted in BJD, pending revision). Here, we further analyze the same patient material, and show that 16 days of sun exposure induces rapid reduction in IFN-α-producing pDCs , $CD11c^{+}CD1c^{-}$ dermal inflammatory DCs as well as well as macrophages in psoriatic skin.

RESULTS

20 patients with psoriasis were enrolled in a study to examine the immunological impact of heliotherapy in psoriatic patients. Clinical and histological data are described in detail elsewhere (Søyland et al., manuscript accepted in BJD, pending revision). Skin biopsies were obtained from 5 randomly selected patients with moderate to severe psoriasis undergoing 16 days of controlled sun exposure in Gran Canaria in the month of March. Specimens were obtained from lesional and non-lesional skin at day 0, day 2 and day 16. All 20 patients experienced clinical improvement with significant reduction in PASI-score during the study period of 16 days. Figure 1 shows PASI-scores for the 5 patients described here. Moreover, epidermal thickening in lesional skin, examined by H+E staining, was significantly reduced in all patients, as described elsewhere (Søyland et al., manuscript accepted in BJD, pending revision).

pDCs and the IFN-α -inducible protein MxA are reduced in lesional dermis after sun exposure

The density of dermal pDCs, defined as cells coexpressing the surface markers CD123 and CD45RA (Figure 2a-e), was markedly higher in lesional compared with non-lesional skin before sun treatment (median 80 vs 2 cells per mm^2). 16 days of sun exposure led to a significant reduction in the density of pDCs in lesional skin ($P<0.05$; Figure 2a, c and

d), whereas a small but significant increase (P<0.05; Figure 2b) was observed in non-lesional skin.

It has been demonstrated that production of IFN-α by pDCs contributes to the pathology of psoriasis (Nestle *et al.*, 2005). We examined formalin-fixed specimens obtained at baseline and day 16 from 5 different patients for the expression of MxA, a well established surrogate marker for IFN-α (Farkas *et al.*, 2001;Fah *et al.*, 1995;Simon *et al.*, 1991). Importantly, we found that whereas MxA was undetectable in non-lesional skin both before and after sun treatment, epidermal expression of MxA was strongly upregulated in lesional epidermis with marked reduction in all patients after sun exposure (Figure 2f-i).

Together, these findings indicated that 16 days of sun exposure significantly lowered the number of pDCs in the lesion coinciding with evidence of reduced IFN-α production.

$CD11c^+CD1c^-$ inflammatory DCs are selectively reduced in lesional dermis after sun exposure

Total numbers of dermal $CD11c^+$ DCs were markedly increased in lesional compared with non-lesional skin (Supplemental Figure 1). In agreement with previous reports (Lowes *et al.*, 2008) we found that this increase was mainly due to a 20-fold increase in the putative inflammatory $CD11c^+CD1c^-$ subset (Fig 3a, c, e and g), of which a substantial fraction coexpressed CD163, DC-SIGN and CD14 (Figures 4a-c and 5a-c). In contrast, the "resident" dermal $CD11c^+CD1c^+$ DC population was only 2-fold increased (Figure 3b, d, e and g). These cells did not express CD163 or DC-SIGN, either in lesional (Figure 6a and b) or in non-lesional dermis (Figure 6c and d). The percentage of $CD11c^+$ DCs expressing the maturation marker DC-LAMP was 2-fold increased in lesional compared with non-lesional skin (Figure 7a-e). As also found by others (Zaba *et al.*, 2009a) DC-$LAMP^+$ cells were mainly located in dermal cellular aggregates, in which most, but not all coexpressed CD1c (Figure 7f).

After 16 days of sun exposure, the $CD11c^+CD1c^-$ DC population in lesional dermis was significantly decreased (P<0.05; Figure 3a, e-f), with marked reduction in cell numbers evident already at day 2, whereas the number of dermal $CD11c^+CD1c^+$ DCs was unchanged (Figure 3b). The percentage of $CD11c^+$ DCs coexpressing DC-LAMP in lesional dermis was also significantly reduced (P<0.05) after sun exposure (Figure 7a, c-d).

As mentioned above, a substantial fraction of $CD11c^+CD1c^-$ DCs expressed monocyte/macrophage markers. The number of $CD11c^+CD1c^-$ DCs coexpressing DC-SIGN decreased ($P<0.05$; Figure 4b) in response to 16 days of sun exposure, whereas the fractions expressing CD163 or CD14 were unchanged (Figure 4a and c).

Although only minor changes in cell densities were observed for the $CD11c^+$ DC subsets in non-lesional skin (Figure 3c and d), small but significant increases in total $CD11c^+$ cells (Supplemental figure 1b) as well as $CD11c^+CD1c^-$ DCs coexpressing CD163, DC-SIGN and CD14 were observed (Figure 4d-f).

Together our findings demonstrated that sun treatment led to a significant reduction of putative inflammatory mDCs as well as a decrease in mature DCs, indicating that a situation more resembling a homeostatic condition was established.

Dermal macrophages are reduced after sun exposure

Differential expression of CD11c and CD163 has been shown to distinguish DCs from macrophages under steady-state conditions (Zaba *et al.*, 2007b). Accordingly, we found that nearly all $CD163^+$ cells in non-lesional skin were negative for CD11c (Figure 4d, 5e and 8d), but expressed DC-SIGN (Figure 5d). A substantial proportion also expressed CD14 (data not shown). In lesional skin a large proportion of putative inflammatory DCs coexpressed several macrophage markers as described above. We therefore defined dermal macrophages as $CD11c^-CD163^+$ cells. This population was approximately 2-fold increased in lesional skin but returned to non-lesional levels after sun treatment (Figure 8a). Also the numbers of $CD11c^-$DC-$SIGN^+$ cells were significantly reduced in lesional dermis after sun exposure ($P<0.05$; Figure 8b), whereas no significant change in $CD11c^-CD14^+$ cell numbers was observed (Figure 8c). Together, this finding demonstrated that dermal macrophages were also significantly affected by sun treatment.

DISCUSSION

It is well documented that UV therapy has beneficial effects in various immune-mediated skin disorders. This effect is believed to be mediated by UV-induced immunosuppression (Ullrich, 2005;Schwarz, 2005). Among cutaneous DCs, epidermal LCs is the best studied subset with respect to the immunosuppressive effect of UV therapy (Ullrich, 2005). However recently, it was shown in a mouse model of contact hypersensitivity that dermal DCs was essential for UV-induced suppression in the absence of LCs (Wang *et al.*, 2009b). We demonstrated here that distinct populations of dermal DCs were dramatically reduced in psoriatic lesions after 16 days of controlled sun exposure. In contrast, we recently found that the number of lesional LCs was only marginally affected examining the same tissue material (Søyland et al., manuscript accepted in BJD, pending revision).

Others have shown that inflammatory $CD11c^{+}CD1c^{-}$ DCs accumulate in psoriatic lesions and drive the inflammatory process by producing IL-23, iNOS, and TNF-α (Lowes *et al.*, 2005;Zaba *et al.*, 2009a). As expected, we also found that $CD11c^{+}CD1c^{-}$ DCs were dramatically increased (20-fold) in lesional skin, and importantly, the number of these cells decreased significantly after 16 days of sun exposure. Moreover, this decrease concurred with reduced mRNA expression for IL-23 (Søyland *et al.,* manuscript accepted in BJD, pending revision). Already after two days we observed reduced numbers of $CD11c^{+}CD1c^{-}$ DCs, which preceded the clinical improvement. The density of "resident" dermal $CD11c^{+}CD1c^{+}$ DCs was unaffected by sun exposure, but the expression of the maturation marker DC-LAMP, primarily found on the $CD11c^{+}CD1c^{+}$ population, was reduced. Our results therefore suggest that sun treatment selectively inhibit accumulation of the inflammatory myeloid subset, allowing the reestablishment of homeostasis by normalizing the balance and maturation status of dermal myeloid DCs.

Recent reports have suggested that accumulating IFN-α-producing pDCs also play a pivotal role in psoriasis. IFN-α has diverse downstream effects, among which are activation of mDCs (Gilliet *et al.*, 2008).We also found that accumulation of pDCs in lesional skin was associated with increased expression of MxA, a protein selectively induced by IFN-α. Importantly, both the number of pDCs and expression of MxA were dramatically reduced in response to sun exposure. To our knowledge, the effect of UV exposure on pDCs has not been examined previously.

The current view of psoriasis immunopathogenesis suggests an intimate crosstalk between stressed keratinocytes, activated pDCs and mDCs, and pathogenic T cells (Lowes *et al.*, 2007). Here and in a separate report (Søyland et al., manuscript accepted in BJD, pending revision) we show that 16 days of sun exposure has a significant favorable effect on both keratinocytes, measured by reduction in epidermal thickness, T cells, and dermal DCs. However, whether reduction in the number and activation state of pDCs and inflammatory mDCs was mainly a direct effect of sun treatment or mediated through the effect on other cells (e.g. T cells and/or keratinocytes) could not be determined in this study. Regulatory T cells are induced by UV therapy (Schwarz *et al.*, 2007). We found a relative increase in the number of $FOXP3^+$ T cells and IL-10 production after treatment (Søyland et al., manuscript accepted in BJD, pending revision) indicating that this immunomodulatory cytokine, produced by regulatory T cells or other cell types could play a role.

There is evidence that macrophages contribute to psoriasis in humans (Marble *et al.*, 2007;Wang *et al.*, 2009a). Here, we showed that although single positive $CD11c^-CD163^+$ and $CD11c^-$DC-$SIGN^+$ macrophages were only 2-fold increased in lesional dermis, these cells were significantly reduced in lesional skin upon sun exposure, suggesting that dermal macrophages may also play a role in this disease.

The psoriatic lesion was dominated by $CD11c^+CD1c^-$ DCs coexpressing the monocyte/macrophage markers CD14, CD163 and DC-SIGN. Recent reports have demonstrated that circulating monocytes are an important source for DCs in peripheral tissues (Auffray *et al.*, 2009). Our phenotypic characterization of inflammatory DCs in psoriatic skin is compatible with this concept. However, the finding that there was a dramatic reduction in the $CD11c^+CD1c^-$ DC population after sun exposure, but no similar decrease in the $CD11c^+$ subset that coexpressed CD163 and CD14 could imply that the $CD11c^+CD1c^-$ DCs represents a distinct subgroup. However, monocytes, macrophages and DCs all show high degree of plasticity and their phenotype and function depend on the local microenvironment (Auffray *et al.*, 2009). Therefore, further studies are needed to define the origin and differentiation pathways of dermal APC populations both under steady state and during inflammation.

Biological agents have received much attention in the treatment of severe psoriasis. Recently, it was shown that etanercept (TNF receptor-immunoglobulin fusion protein) had a significant effect on psoriasis by inhibiting the activity of dermal DCs (Zaba *et al.*, 2007a). Importantly, we show here, and in a separate report (Søyland et al), that sun

treatment has at least comparable effect with two weeks of etanercept treatment based on the reduction of PASI-score, epidermal thickness, $CD11c^+$ DCs, $CD163^+$ macrophages, $CD3^+$ T cells, MxA expression, DC-LAMP expression and IL-23 mRNA.

In conclusion, this study demonstrates that sun exposure results in rapid reductions in pathogenic DC subsets as well as macrophages in psoriatic lesional dermis, thus restoring conditions associated with homeostasis. These changes took place preceding clinical improvement, suggesting that UV-induced immunosuppression is mediated, at least in part, through an effect on dermal APCs and their products.

MATERIALS AND METHODS

Study population.

A total of 20 patients (mean/median age 47.2/48 years, range 24-65, 6 females and 14 males) were enrolled in the study and transported from Norway to Gran Canary, Spain, in the month of March. The patients were evaluated by the same dermatologist for the Psoriatic Area and Severity Index (PASI) (Fredriksson and Pettersson, 1978) before and after heliotherapy. All patients had moderate to severe plaque psoriasis, i.e. mean/median PASI before sun exposure of 9.8/8.7, range 3.8-18.8. All patients had stopped using any psoriasis medication at least 4 weeks prior to this study. Two of the patients had skin type II and 18 had skin type III according to the Fitzpatrick classification (Fitzpatrick, 1988).

UV was measured every hour from 9 a.m. to 5 p.m. using broadband UVB and UVA plus narrowband 311 nm broadband CIE-weighted UVB and spectral UVA-detectors as described elsewhere (Nilsen *et al.*, 2009)

The study was approved by the Regional Committee of Medical Ethics and all patients gave their written, informed consent.

Clinical data for all 20 patients are presented elsewhere (Søyland et al., manuscript accepted in BJD, pending revision).

Skin biopsies

4 mm punch biopsy specimens from lesional and non-lesional skin from 5 patients on days 0 (baseline), 2 and 16 and from another 5 patients on day 0 and 16 (used here for MxA detection). The samples from each individual patient were obtained within the

same body area on all days but in sufficient distance to avoid reactive inflammation from prior biopsy sampling. Specimens were either snap frozen in liquid nitrogen and stored at -70˚ or fixed in formalin and thereafter embedded in paraffin.

Immunohistochemistry

Frozen sections were cut at 4 μm and fixed in acetone prior to staining. Two-colour immunofluorescence staining was performed as detailed elsewhere (Søyland et al., manuscript accepted in BJD, pending revision). The panel of antibodies and reagents used are given in Table S1.

MxA staining was performed on formalin-fixed and paraffin-embedded specimens, because this antibody does not work on frozen sections. Details are described elsewhere (Farkas *et al.*, 2001).

Stained sections were examined blindly by fluorescence microscopy at x 400 magnification by the same investigator (IH). An ocular grid was used and all positive immunostained cells in the dermis to a depth of 250 μm below the the rete ridges. Cell counts were given as total number per square millimetre. MxA staining of the epithelium was estimated on an arbitrary scale from 0 to 5.

Statistics

Non-parametric Friedman test with Dunn's multiple comparison post-test was performed to compare cell counts obtained from 5 different patients at three different time-points. Student's t-test was used for analysis of PASI-score. $P<0.05$ was interpreted as significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. PASI scores were significantly reduced in patients after 16 days of sun exposure.

All 5 patients included in this study showed excellent clinical improvement after 16 days of sun exposure with significant reductions in PASI-scores (Student's t-test).

Figure 2. The numbers of pDCs and IFN-α expression in lesional skin were reduced by sun exposure.

The number of pDCs, defined as cells expressing both CD123 (red images) and CD45RA (green images), was markedly increased in lesional compared with non-lesional dermis **(a** and **b)** and was significantly reduced in lesional dermis after 16 days of sun exposure **(a, c** and **d)**. In contrast, a significant increase in pDCs was seen in non-lesional skin after sun exposure. Note that the anti-CD123 antibody (red) stains endothelial cells **(d-f)**.

In non-lesional skin, expression of MxA, a surrogate marker for IFN-α expression, was markedly upregulated in lesional skin whereas it was undetectable in non-lesional skin **(f, g** and **i)**. After 16 days of sun exposure, MxA expression was markedly reduced in lesional skin **(f** and **h)** The increase in pDC numbers after sun exposure in non-lesional skin was not paralleled by an increase in MxA-staining **(b** and **f)**. All images are at x200 magnification. Bars=25 μm

Figure 3. $CD11c^+$ $CD1c^-$DC populations lesional dermis are rapidly and selectively reduced upon sun exposure

The putative inflammatory $CD11c^+CD1c^-$ DCs were 20-fold increased at baseline in lesional dermis **(a)** compared with non-lesional **(c).** This cell population was significantly reduced at day 16 of sun exposure with marked reductions evident already at day 2 **(a, e** and **f)**. $CD11c^+CD1c^+$ DCs, i.e. putative homeostatic DCs were only 2-fold increased in lesional dermis and were unchanged after sun exposure **(b, e** and **f)**. In non-lesional dermis only very few $CD11c^+$CD1c- cells were found at baseline **(c** and **g)**. No significant changes in $CD11c^+CD1c^-$ or $CD1c^+$ DCs were seen in lesional dermis after sun exposure **(c** and **d).** Images are at x200 magnification. Bars=25 μm

Figure 4. In lesional dermis a substantial fraction of CD11c$^+$ DCs coexpress the monocyte/macrophage markers CD163, DC-SIGN and CD14. The number of CD11c$^+$ DCs coexpressing the monocyte/macrophage markers CD163 **(a)**, DC-SIGN **(b)** and CD14 **(c)** were markedly higher in lesional than in non-lesional dermis where only very few double positive cells could be found **(d-f)**. Only the number of CD11c$^+$DC-SIGN$^+$ cells was significantly reduced after 16 days of sun exposure **(b)**. In non-lesional dermis sun exposure induced small but significant increases in all these three subsets **(d-f)**.

Figure 5. CD11c$^+$ DCs in lesional skin coexpress monocyte/macrophage markers. Immunohistochemical staining shows the infiltration of CD11c$^+$ cells (green images) coexpressing the monocyte/macrophage markers CD163 **(a)**, DC-SIGN **(b)** and CD14 **(c)** (all red images). In non-lesional dermis only occasional CD11c$^+$ DCs coexpressed CD163 **(e)**, DC-SIGN **(f)** or CD14 **(g)**. DC-SIGN was primarily expressed on CD163$^+$ cells in both lesional **(d)** and non-lesional skin **(h)**. Images are at x400 magnification. Bars=50μm

Figure 6. CD1c$^+$ DCs did not express CD163 or DC-SIGN

CD1c$^+$ DCs (red images) accumulate in dermal cellular aggregates in lesional dermis **(a** and **b)**. No CD1c$^+$ DCs coexpressed CD163 or DC-SIGN at baseline in lesional dermis **(a** and **b)** or non-lesional **(c** and **d)**. Images are at x400 magnification. Bars=50μm

Figure 7. Expression of DC maturation marker DC-LAMP (CD208) was reduced in bothlesional and non-lesional dermis after sun exposure

The percentage of dermal CD11c$^+$ cells (green images) that coexpressed the maturation marker DC-LAMP (red images) was approximately 2-fold increased in lesional skin **(a** and **c)** compared with non-lesional **(b** and **e)** at baseline. After 16 days of sun exposure significant reductions in this percentage was seen in both lesional **(a** and **c)** and non-lesional dermis **(b)**. DC-LAMP$^+$ cells (**f**, green image) accumulated in dermal cellular aggregates, with CD1c$^+$ cells (**f**, red image) and most, but not all DC-LAMP$^+$ cells were CD1c$^+$ **(f)**. Images **(c-e)** are at x200 magnification, bars=25μm. Image **(f)** is at x400 magnification, bar=50μm

Figure 8. The numbers $CD11c^{-}CD163^{+}$ and $CD11c^{-}$DC-$SIGN^{+}$ macrophages were significantly reduced in lesional dermis after sun exposure. In lesional dermis single positive (i.e. $CD11c^{-}$) $CD163^{+}$ **(a)** and DC-$SIGN^{+}$ macrophages **(b)** were approximately 2-fold increased compared with non-lesional dermis (**d** and **e)** and significantly reduced in after sun exposure. Single positive $CD14^{+}$ cells **(c)** were also 2-fold increased in lesional dermis compared with non-lesional **(f)** with no significant change upon sun exposure. No significant changes in any of these cell subsets were observed in non-lesional dermis **(d-f)**.

Supplemental Figure 1. The total number of $CD11c^{+}$ DCs was unchanged in lesional dermis after sun exposure. The total number of $CD11c^{+}$ cells was markedly increased in lesional **(a)** compared with non-lesional dermis **(b).** In lesional dermis, numbers were reduced in all individuals but the change was non-significant **(a).** In non-lesional dermis a small but significant increase in total $CD11c^{+}$ numbers was seen at day 16 of sun exposure **(b)**.

Supplemental Table 1. List of primary and secondary antibodies and reagents with manufacturers used in this paper.

Figure 1

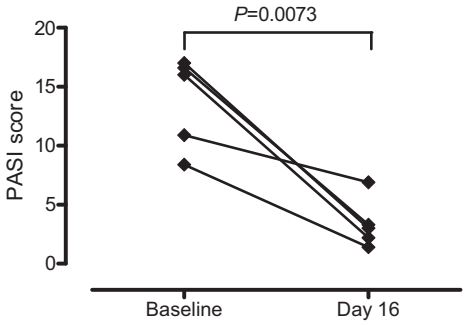


Figure 2

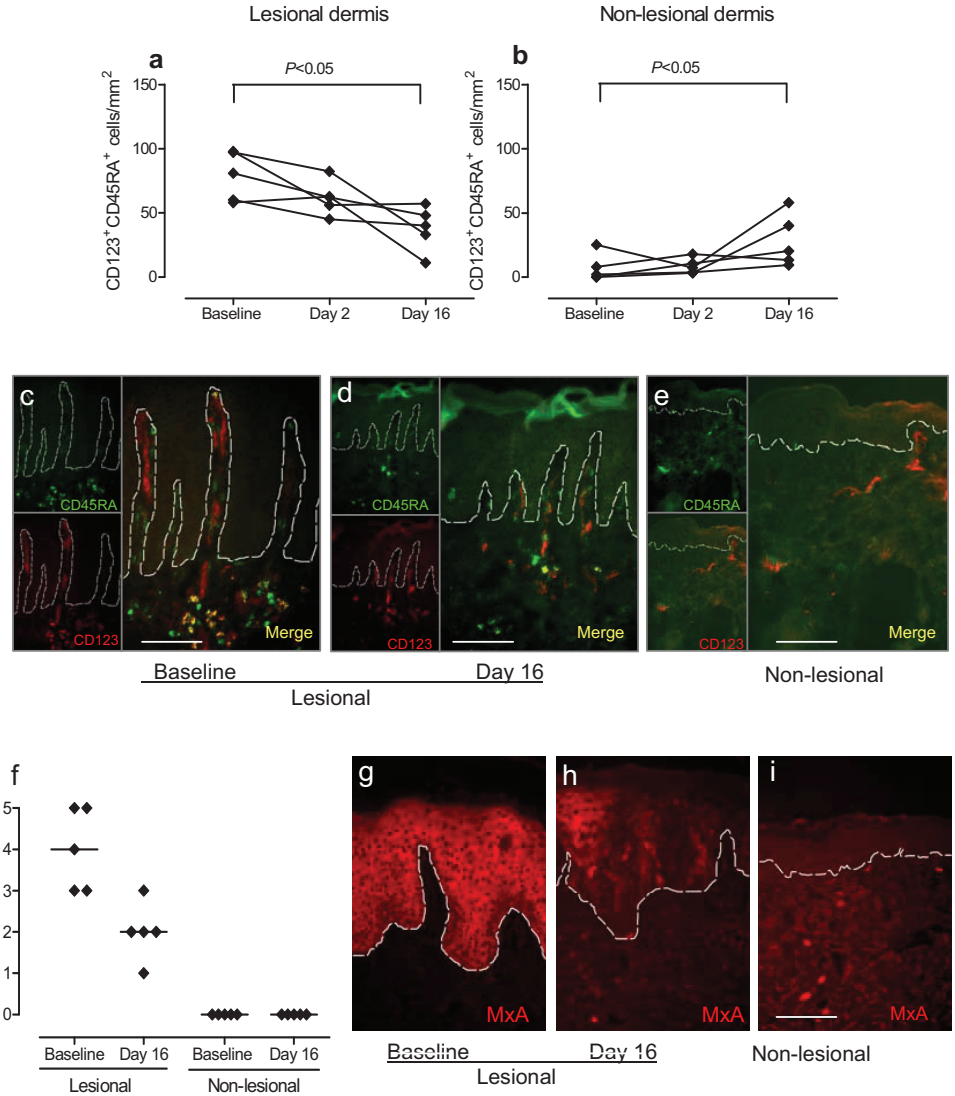

Lesional dermis
Non-lesional dermis
a
b
P<0.05
P<0.05
CD123+CD45RA+ cells/mm2
150
100
50
0
Baseline
Day 2
Day 16
c
d
e
CD45RA
CD123
Merge
Baseline
Day 16
Lesional
Non-lesional
f
MxA expression
5
4
3
2
1
0
Baseline
Day 16
Baseline
Day 16
Lesional
Non-lesional
g
h
i
MxA
Baseline
Day 16
Lesional
Non-lesional

Figure 3

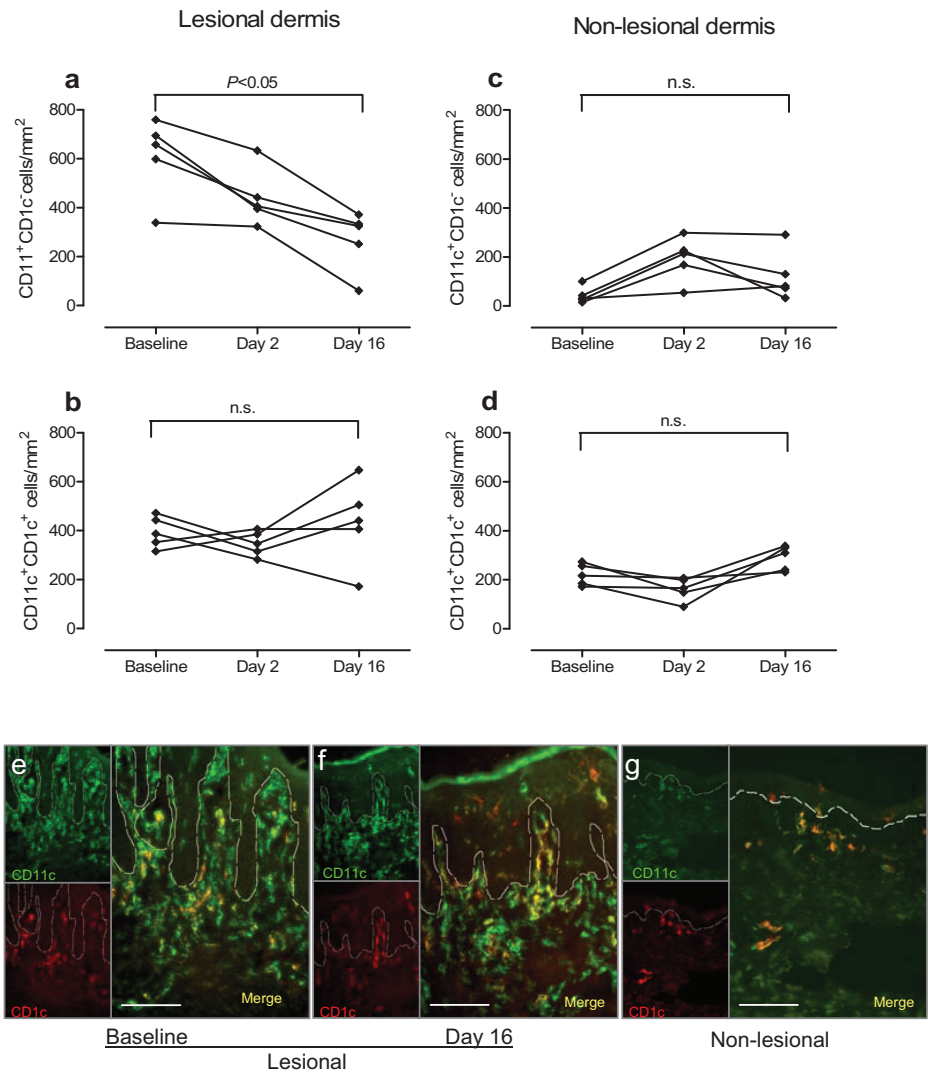

Lesional dermis
Non-lesional dermis
a
b
c
d
P<0.05
n.s.
n.s.
n.s.
CD11+CD1c-cells/mm2
CD11c+CD1c- cells/mm2
CD11c+CD1c+ cells/mm2
800
600
400
200
0
Baseline
Day 2
Day 16
e
f
g
CD11c
CD1c
Merge
Baseline
Day 16
Lesional
Non-lesional

Figure 4

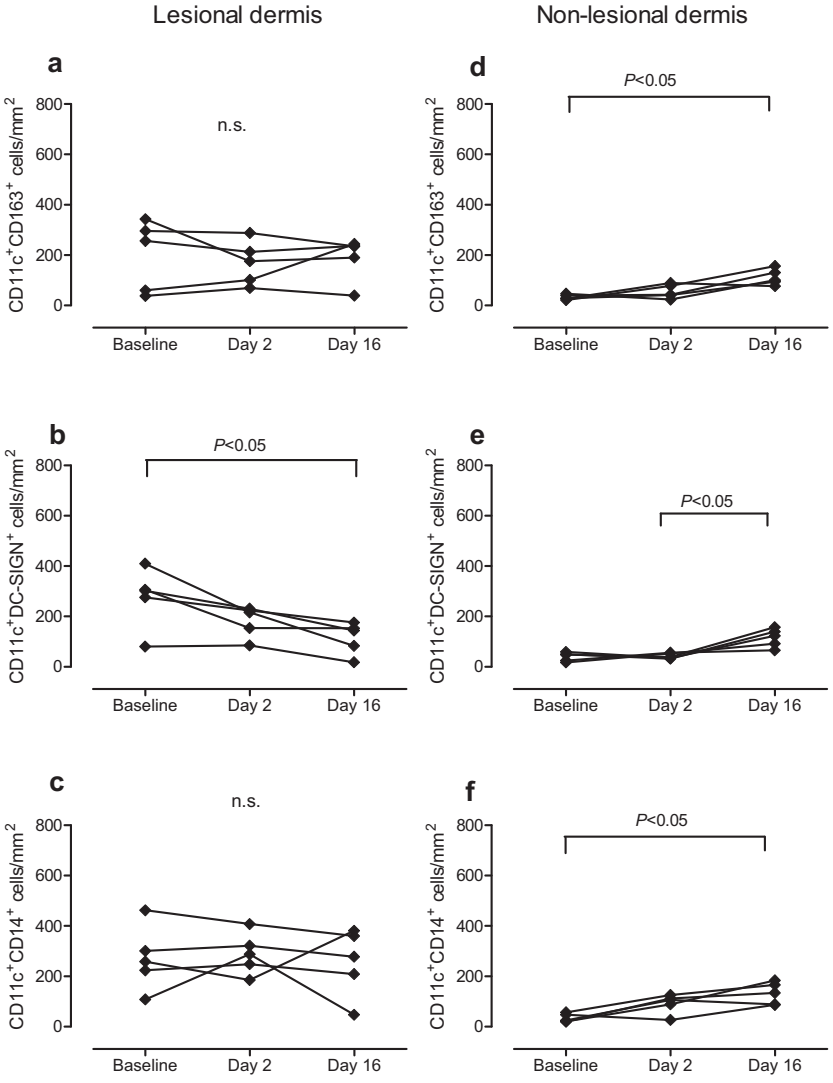

Lesional dermis
Non-lesional dermis
a
b
c
d
e
f
n.s.
P<0.05
CD11c+CD163+ cells/mm2
CD11c+DC-SIGN+ cells/mm2
CD11c+CD14+ cells/mm2
0
200
400
600
800
Baseline
Day 2
Day 16

Figure 5

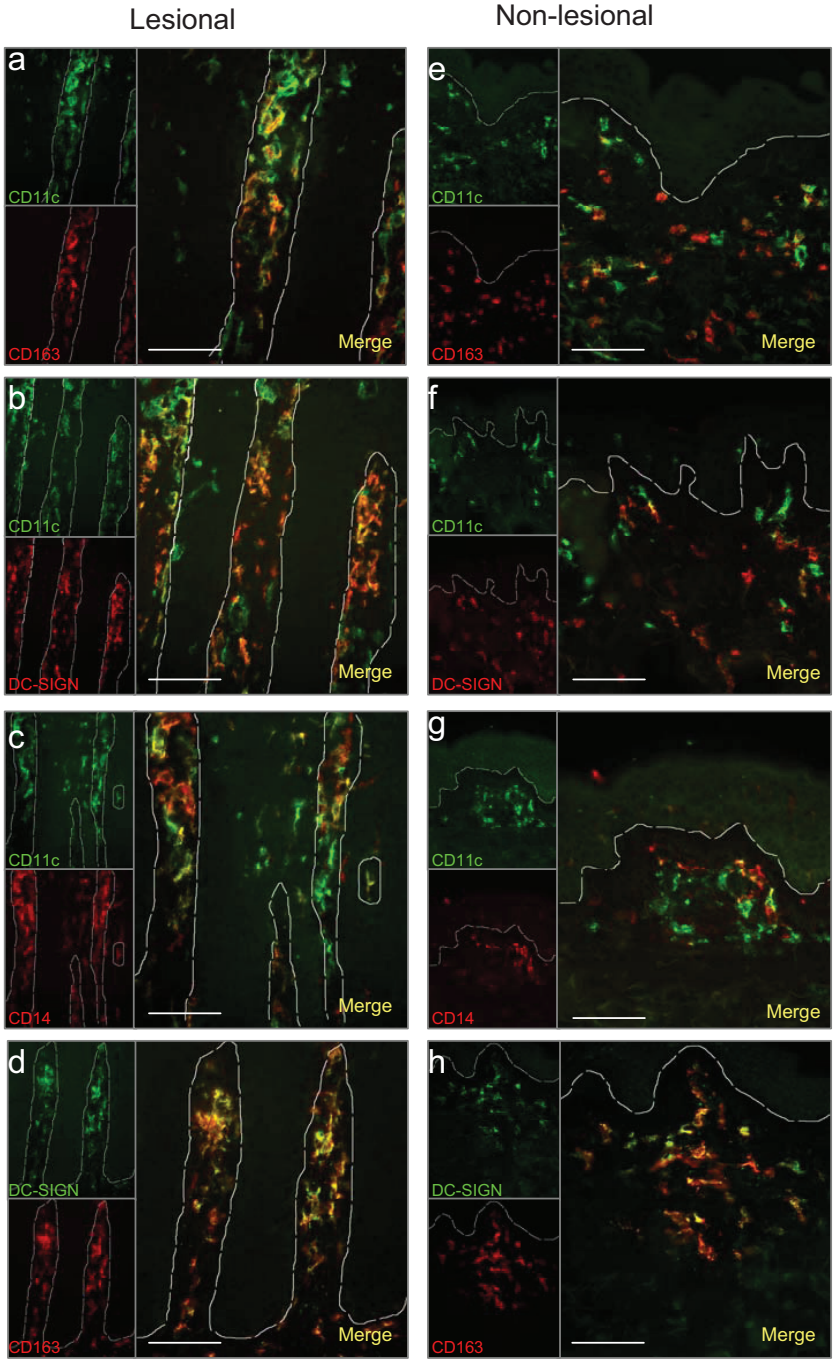

Lesional
Non-lesional
a
CD11c
CD163
Merge
e
CD11c
CD163
Merge
b
CD11c
DC-SIGN
Merge
f
CD11c
DC-SIGN
Merge
c
CD11c
CD14
Merge
g
CD11c
CD14
Merge
d
DC-SIGN
CD163
Merge
h
DC-SIGN
CD163
Merge

Figure 6

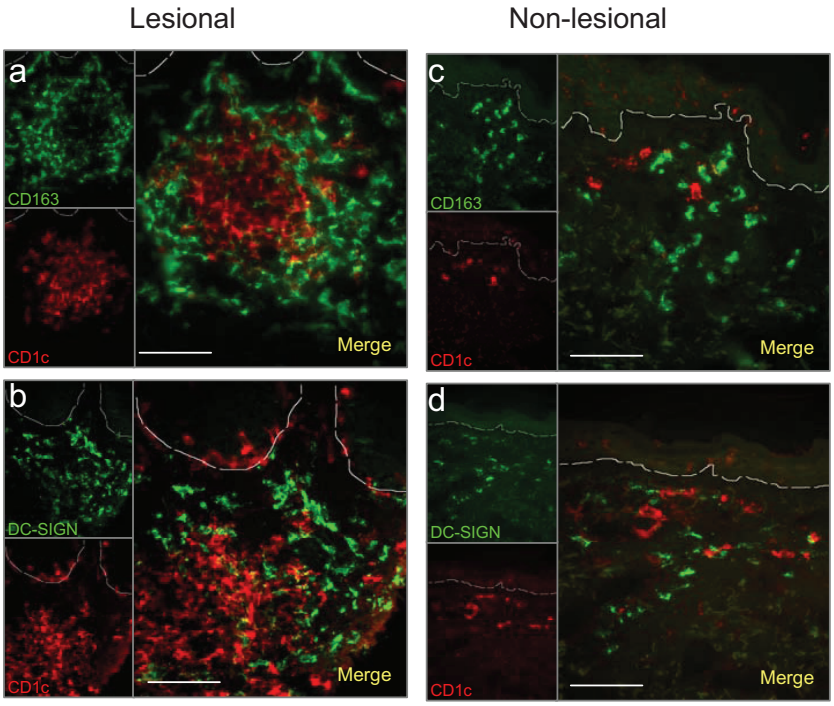

Lesional
Non-lesional
a
CD163
CD1c
Merge
c
CD163
CD1c
Merge
b
DC-SIGN
CD1c
Merge
d
DC-SIGN
CD1c
Merge

Figure 7

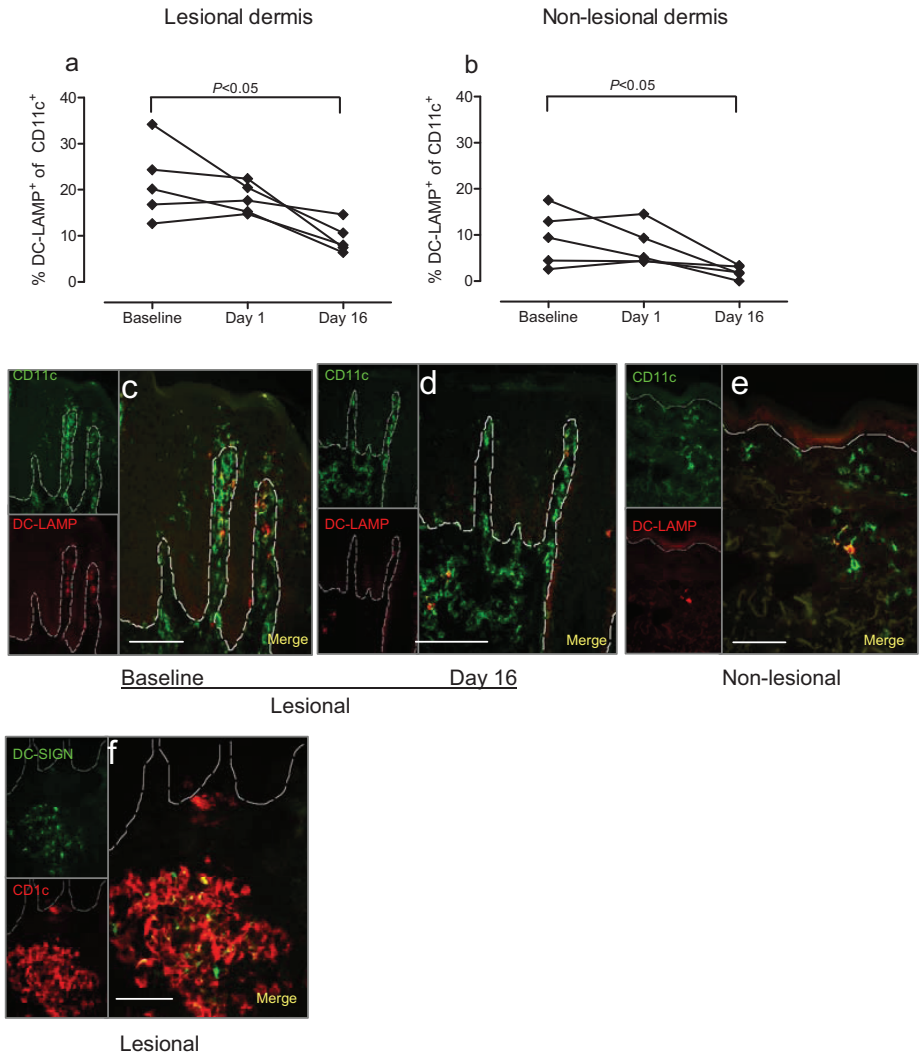
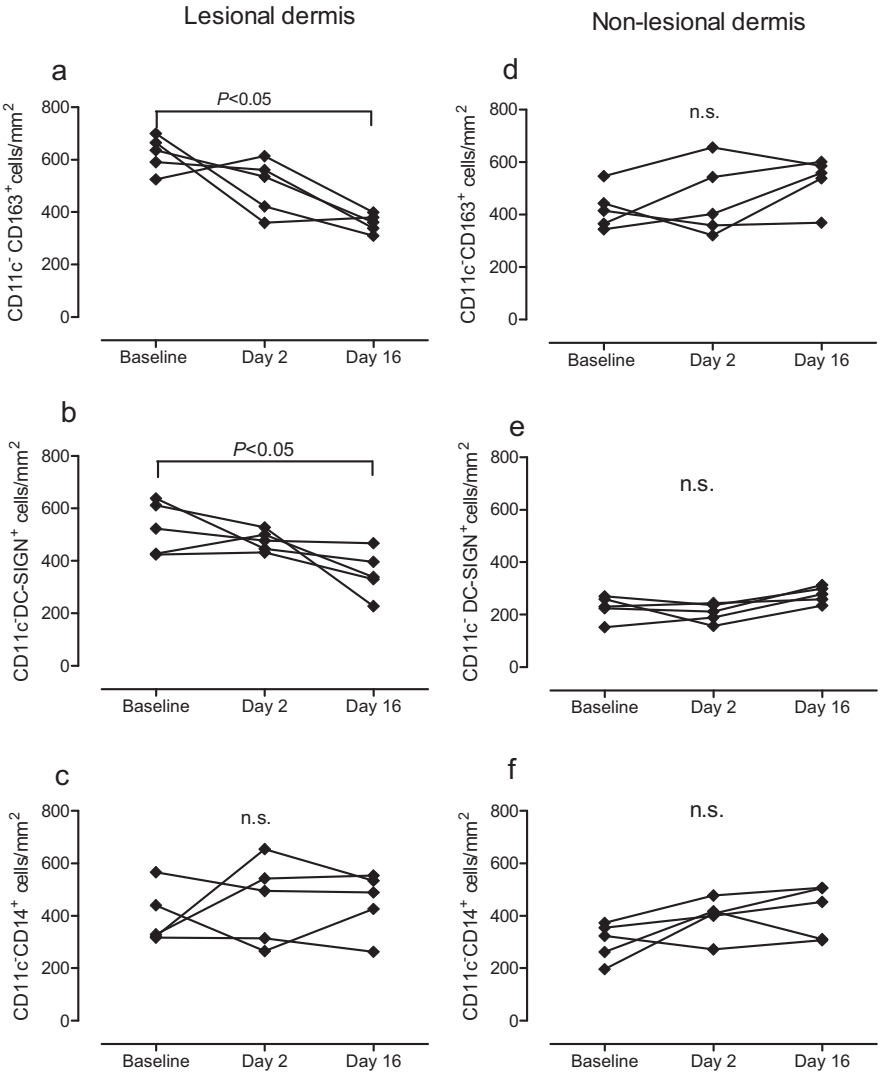

Lesional dermis
Non-lesional dermis
a
b
P<0.05
P<0.05
% DC-LAMP+ of CD11c+
40
30
20
10
0
Baseline
Day 1
Day 16
CD11c
DC-LAMP
Merge
c
d
e
Baseline
Day 16
Lesional
Non-lesional
DC-SIGN
CD1c
f
Lesional

Figure 8


Lesional dermis
Non-lesional dermis
a
b
c
d
e
f
P<0.05
n.s.
CD11c-CD163+cells/mm2
CD11c-DC-SIGN+cells/mm2
CD11c-CD14+cells/mm2
CD11c-CD163+ cells/mm2
CD11c- DC-SIGN+cells/mm2
CD11c-CD14+ cells/mm2
0
200
400
600
800
Baseline
Day 2
Day 16

Supplemental figure 1

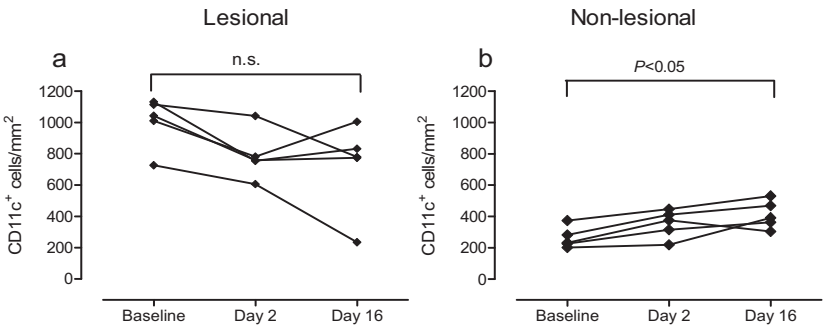


Table S1

Primary antibodies			
Antibody specificity	**Clone**	**Isotype**	**Manufacturer**
HLA-DR	L243	IgG2a	BD Immunocytometry Syst., San Jose, CA
CD11c	CBR-p150/4G1	IgG2a	Biosource, Camarillo, CA
CD11c	KB90	IgG1	Gift from Dr. Pulford, Oxford
CD1c	M241	IgG1	Ancell, Bayport, MN
CD163	RM3/1	IgG1	Affinity Bioreagents, Golden, CO
CD163	GHI/61	IgG1-biotin	BioLegend, San Diego, CA
DC-SIGN (CD209)	516.111	IgG2a	Gift from Dr. Westin, Oslo, Norway
DC-SIGN (CD209)	531.111	IgG1	Gift from Dr. Westin, Oslo, Norway
CD14	RM052	IgG2a	Biosys, Compiegne, France
DC-LAMP (CD208)	104G4	IgG1	Immunotech, Marseille, FR
DC-LAMP (CD208)	104G4	IgG1-biotin	Nordic Biosite, Täby, Sweden
CD45RA	L48	IgG1	BD Immunocytometry Syst., San Jose, CA
CD123	7G3	IgG2a	BD Pharmingen, San Diego, CA
MxA	M143	IgG2a	Gift from Dr. Haller, Freiburg, Germany
Secondary antibodies			
Antibody specificity	**Origin**	**Labelling**	**Manufacturer**
Mouse IgG1	Goat	Cy3	Southern Biotech, Birmingham, AL
Mouse IgG2a	Goat	biotin	Southern Biotech, Birmingham, AL
Mouse IgG (H+L)	Goat	Cy3	Jackson Immunoresearch, West Grove, PA
Mouse IgG1	Goat	biotin	Southern Biotech, Birmingham, AL
Mouse IgG2a	Goat	Cy3	Southern Biotech, Birmingham, AL
Streptavidin		Cy2	Amersham Biosciences, Little Chalfont, UK

Errata:

The following changes have been made in the manuscript Paper II since the thesis was originally submitted for evaluation. The doctoral committee has granted permission for the changes to be implemented in the current printed version.

Page 6, line 15: deleted “not shown”.
Page 7, line 5: inserted “(Figures 4B and C)”
Page 7, line 10: “5A” replaced by “4C”
Page 7, line 20: “5B” replaced by “5”
Page 7 line 22: “5B” replaced by “5”

Page 2, abstract, line 11: ”mm” replaced by “cm”
Page 6, line 27: ”mm” replaced by “cm”
Page 8, line 32: ”mm” replaced by “cm”
Page 9, line 2: ”mm” replaced by “cm”